PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/705, C12N 15/62, 1/21, A61K 38/17 // (C12N 1/21, C12R 1:19)

A1

(11) International Publication Number: WO 95/01994

(43) International Publication Date: 19 January 1995 (19.01.95)

(21) International Application Number: PCT/US94/07685

(22) International Filing Date: 8 July 1994 (08.07.94)

(30) Priority Data:
08/090,533 9 July 1993 (09.07.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/090,533 (CIP)
Filed on 9 July 1993 (09.07.93)

(71) Applicant *(for all designated States except US)*: SYNERGEN, INC. [US/US]; 1885 33rd Street, Boulder, CO 80301 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: COX, George, N. [US/US]; 678 West Willow Street, Louisville, CO 80027 (US). PRATT, Dickson, G. [US/US]; 4489 Wellington Road, Boulder, CO 80301 (US).

(74) Agents: KOIVUNIEMI, Paul, K. et al.; Synergen, Inc., 1885 33rd Street, Boulder, CO 80301 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECOMBINANT CTLA4 POLYPEPTIDES AND METHODS FOR MAKING THE SAME

(57) Abstract

The present invention relates to recombinantly-produced CTLA4 polypeptides having B7 binding activity that are not the product of a fusion between CTLA4 and human Ig genes. The CTLA4 polypeptides of the present invention generally contain an amino acid sequence corresponding to the extracellular domain of the CTLA4 receptor protein. The invention further provides functional derivatives of the CTLA4 polypeptides, including muteins and conjugates containing polyethylene glycol. Methods for preparing the recombinant CTLA4 polypeptides are also provided, as well as methods for separating the monomeric and dimeric forms of the recombinant polypeptides as well as active and less active dimer forms.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/705, C12N 15/62, 1/21, A61K 38/17 // (C12N 1/21, C12R 1:19)

A1

(11) International Publication Number: WO 95/01994

(43) International Publication Date: 19 January 1995 (19.01.95)

(21) International Application Number: PCT/US94/07685

(22) International Filing Date: 8 July 1994 (08.07.94)

(30) Priority Data:
08/090,533 9 July 1993 (09.07.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/090,533 (CIP)
Filed on 9 July 1993 (09.07.93)

(71) Applicant *(for all designated States except US)*: SYNERGEN, INC. [US/US]; 1885 33rd Street, Boulder, CO 80301 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: COX, George, N. [US/US]; 678 West Willow Street, Louisville, CO 80027 (US). PRATT, Dickson, G. [US/US]; 4489 Wellington Road, Boulder, CO 80301 (US).

(74) Agents: KOIVUNIEMI, Paul, K. et al.; Synergen, Inc., 1885 33rd Street, Boulder, CO 80301 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECOMBINANT CTLA4 POLYPEPTIDES AND METHODS FOR MAKING THE SAME

(57) Abstract

The present invention relates to recombinantly-produced CTLA4 polypeptides having B7 binding activity that are not the product of a fusion between CTLA4 and human Ig genes. The CTLA4 polypeptides of the present invention generally contain an amino acid sequence corresponding to the extracellular domain of the CTLA4 receptor protein. The invention further provides functional derivatives of the CTLA4 polypeptides, including muteins and conjugates containing polyethylene glycol. Methods for preparing the recombinant CTLA4 polypeptides are also provided, as well as methods for separating the monomeric and dimeric forms of the recombinant polypeptides as well as active and less active dimer forms.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

RECOMBINANT CTLA4 POLYPEPTIDES AND METHODS FOR MAKING THE SAME

The present invention relates to soluble proteins useful for controlling T cell activation, and more particularly to soluble CTLA4 proteins produced by recombinant DNA methods.

Background of the Invention

Inappropriate T cell activation has been implicated in a number of deleterious conditions, including autoimmune diseases and transplant rejection. Optimal activation of T cells for clonal expansion is believed to require two signals. One is an antigen-specific signal delivered through T cell receptors (TCR), while the second is an antigen-non-specific or co-stimulatory signal. Chen et al., Cell 71:1093-1102 (1992); Liu et al., Eur. J. Immunol. 22:2855-2859 (1992).

The second non-specific signal is determined by a class of T cell regulatory molecules known as co-stimulators that determine whether T cells are activated to proliferate or enter into a state of unresponsiveness known as clonal anergy. B7, a T cell regulatory molecule, is a co-stimulatory protein expressed on the cell surface of antigen presenting cells such as activated macrophages, activated B lymphocytes and dendritic cells as reported in Razi-Wolf et al., Proc. Natl Acad. Sci. U.S.A. 89:4210-4214 (1992) and Freeman et al., J. Immunol. 139:326-3267 (1992).

B7 is a natural ligand for T cell surface proteins known as CD28 and CTLA4 (cytolytic T-lymphocyte-associated antigen number 4). CD28 and CTLA4 share substantial homology in their amino acid sequences, particularly in the transmembrane and cytoplasmic domains, and are therefore believed to share similar functions in the T-cell co-stimulation pathway. B7 is known to have a greater affinity for CTLA4 compared with CD28.

CD28 is constitutively expressed on most T lymphocytes. CTLA4, however, was determined to be preferentially expressed by activated versus unactivated cytolytic T cells in DNA hybridization experiments described in Brunet et al., Nature 328:267-270 (1987). It is now known that CTLA4 is expressed by

activated cytotoxic T lymphocytes and activated helper T lymphocytes.

The interactions of B7 with CD28 and CTLA4 play an important role in the full activation of T cells in the co-stimulation pathway during an immune response. Neutralization of B7 or CD28 activity, for example with monoclonal antibodies, prevents T cell proliferation in response to foreign antigens and polyclonal activators such as lectins. Neutralization of B7 activity also prevents T cells from acting as helper cells for the induction of antibody synthesis by B cells.

In addition to playing an important role in T cell proliferation and antibody induction, the interaction of B7 with CD28 regulates cytokine synthesis in T lymphocytes. Cytokines that are known to be regulated by the interaction of B7 with CD28 include interleukin-2, tumor necrosis factors alpha and beta, gamma interferon and granulocyte-macrophage colony stimulating factor (Gimmi et al., Proc. Nat'l Acad. Sci. U.S.A. 88:6575-6579 (1991); Linsley et al., J. Exp. Med. 173:721-730 (1991); and Thompson et al., Proc. Nat'l Acad. Sci. U.S.A. 86:1333-1337 (1989)).

Synthesis of these cytokines is not completely inhibited by commonly used immunosuppressive agents such as cyclosporine, which is a fungal metabolite used to suppress the immune system in patients undergoing organ transplants or suffering from autoimmune diseases. Consequently, it is believed agents that effectively inhibit B7 activity could be used as an alternative to cyclosporine therapy or in combination with cyclosporine to provide an additive or synergistic effect in inhibiting T cell proliferation. Because of the similarity between CD28 and CTLA4, it is believed that the interaction of B7 with CTLA4 should also regulate cytokine synthesis in T lymphocytes.

However, previous attempts to express the extracellular domain of CTLA4 as a soluble, unfused protein have been unsuccessful. According to PCT Publication No. WO 93/00431, successful expression of active CTLA4 proteins is believed to require an expression system that permits the proteins to form as dimers because the proteins are believed to occur in nature as

dimers. Thus, researchers have focussed on fusion proteins in an effort to find an appropriate expression system.

A fusion protein containing the extracellular domain of CTLA4 joined to the F_c heavy chain region of an immunoglobulin molecule has been developed as a possible agent having B7 inhibitory activity. This fusion protein, referred to as "CTLA4-Ig fusion protein," is described in Linsley et al., J. Exp. Med. 174:561-569 (1991) and in PCT Patent Publication No. WO 93/00431. According to these publications, the CTLA4-Ig fusion protein is secreted from mammalian cells as a disulfide-linked dimeric protein that aggregates in solution. However, attempts to express the extracellular domain of CTLA4 as an unfused protein in mammalian cells were unsuccessful. The Ig portion of the fusion protein facilitates the formation of a dimeric fusion protein, which is capable of being processed and expressed by the mammalian cells. The Ig portion additionally allows the active fusion protein to be purified from conditioned media using a protein A affinity column. Protein A has a high affinity for the F_c region of Ig molecules.

The molecular weight of the major CTLA4-Ig species in solution is approximately 200 kDa based on size exclusion chromatography also described in Linsley et al., supra. Because the molecular weight of the monomeric form of the CTLA4-Ig fusion protein expressed in mammalian cells is about 50 kDa, the major species in solution is believed to be an aggregated complex of at least four CTLA4-Ig molecules.

The B7 inhibitory activity of the CTLA4-Ig fusion protein has been tested in both *in vitro* and *in vivo* experiments. In the *in vitro* experiments, the CTLA4-Ig fusion protein bound to B7 and neutralized its activity. In fact, the CTLA4-Ig fusion protein was a better inhibitor of B7 activity than a comparable CD28-Ig fusion protein. The results of these assays are consistent with the previous experiments showing that B7 binds to CTLA4 with greater affinity than CD28. The CTLA4-Ig fusion protein was found to inhibit T cell proliferation, with a half maximal inhibitory dose of 30 ng/ml, in a mixed lymphocyte reaction as reported in Linsley et al., supra. The fusion protein also

inhibited the ability of helper T cells to stimulate antibody production by B lymphocytes in an *in vitro* study described in Linsley et al., J. Exp. Med. 174:561-569 (1991).

In experiments conducted *in vivo*, the CTLA4-Ig fusion protein was determined to be immunosuppressive and capable of prolonging survival of pancreatic and heart allografts in mice and rats (Lenschow et al., Science 257:789-792 (1992) and Turka et al., Proc. Nat'l Acad. Sci. U.S.A. 89:11102-11105 (1992)). In the mouse study, the administration of CTLA4-Ig resulted in the long term acceptance of pancreatic allografts. These results suggest that the fusion protein had tolerized the recipient mice to the foreign tissue. Other animal studies described in Linsley et al., Science 257:792-795 (1992) also demonstrate that CTLA4-Ig was capable of inhibiting primary antibody responses to foreign antigens such as sheep red blood cells.

The CTLA4-Ig fusion protein has several disadvantages as a therapeutic agent for human disease. Because the fusion protein is a non-naturally occurring molecule, a patient receiving the protein may develop an immune response to the protein. Antigenicity may be more of a problem when patients are taken off the therapeutic agent so they are no longer immunosuppressed and are capable of mounting an immune response against the fusion protein. Therefore, antigenicity may prevent the CTLA-Ig fusion protein from being administered intermittently to patients suffering from chronic diseases. In addition, the half-life of the CTLA4-Ig fusion protein in mice is about 4 days, with significant levels of the fusion protein still detectable in the animals 5 weeks after the cessation of treatment with CTLA4-Ig. Linsley et al., Science 257:792-795 (1992). Furthermore, when bound to B7 on the surface of antigen-presenting cells, the Ig portion of the fusion protein may activate the complement cascade that results in cell death and hematological problems. Finally, CTLA4-Ig fusion protein is expressed in mammalian cells, which is a costly method of producing recombinant proteins.

Thus, a need exists for additional therapeutic agents capable of inhibiting the co-stimulatory pathway in T cell

activation. The present invention satisfies this need and provides related advantages as well.

Summary of the Invention

The present invention relates to recombinantly-produced CTLA4 polypeptides that are not fusion proteins containing human Ig molecules. The soluble, recombinant polypeptides contain, as a basic unit, a monomer consisting essentially of the extracellular domain of the CTLA4 receptor protein. Preferably, the recombinant polypeptides are the product of joining two or more monomers through intermolecular disulfide bonds or through a cross-linking agent, such as polyethylene glycol (PEG), to form biologically active dimers and other multimers.

The polypeptides can also be functional derivatives of the monomers and multimers, such as cysteine muteins in which a cysteine is substituted for one or more amino acids or is added to the wild-type CTLA4 amino acid sequence. The substitution is preferably made at residue numbers 79, 80, 81, 109, 110 or 111 of the extracellular domain of the CTLA4 receptor protein as shown in SEQ ID NO.2, while the addition of an extra cysteine is preferably made after residue number 125 from the N-terminal end of the naturally-occurring CTLA4 receptor protein. Other functional derivatives include, for example, pegylated dumbbell molecules in which two cysteine muteins are attached through activated groups on each end of a PEG molecule.

The present invention also provides methods of making the recombinant polypeptides. The methods include the steps of:

(a) obtaining a DNA sequence capable of directing a host cell to express a polypeptide corresponding to the extracellular domain of a CTLA4 receptor protein, the polypeptide having B7 binding activity;

(b) inserting the DNA sequence into a vector having operational elements for expression of the DNA sequence;

(c) transferring the vector into a host cell capable of expressing the polypeptide;

(d) culturing the host cell under conditions for expression of the polypeptide;

(e) harvesting the polypeptide; and

(f) permitting the polypeptide to assume an active tertiary structure. Optionally, the polypeptide can also be permitted to assume an active quaternary structure.

Vectors and host cells useful for the expression of the recombinant CTLA4 polypeptides are also provided. In addition, the invention further provides pharmaceutical compositions containing the CTLA4 polypeptides as the active ingredient.

The invention also relates to methods for separating the various forms of the recombinantly-produced polypeptides, particularly the separation of monomers and dimers. The invention also relates to methods for separating various dimer species and purifying active dimers from less active dimers. After obtaining active, recombinant polypeptides according to the above methods, the resulting mixture is passed over an ion exchange column, particularly an anion exchange column, followed by passage over a sizing column. The separation methods produce one pool mixture of at least about 90% dimers and a second pool of at least about 85% monomers. These methods also separate active dimers from less active dimers.

Detailed Description of the Invention

The present invention provides soluble, recombinantly-produced CTLA4 polypeptides that are not fused to human Ig molecules. The novel recombinant polypeptides of the present invention are useful for inhibiting inappropriate T cell proliferation that can lead to various disorders.

The naturally-occurring or wild-type CTLA4 protein is a ligand of B7, which is a cell surface protein involved in the co-stimulatory pathway leading to T cell activation. The nucleotide and amino acid sequences of murine and human CTLA4 are reported in Brunet et al., Nature 328:267-270 (1987) and Dariavach et al., Eur. J. Immunol. 18:1901-1905 (1988), respectively. The overall amino acid homology between human and murine CTLA4 proteins is 76%. The correct amino acid sequence of the full length human CTLA4 protein is provided in PCT Publication No. WO 93/00431, published on January 7, 1993.

As noted previously, earlier attempts to produce an unfused or truncated CTLA4 protein have been unsuccessful. Therefore, prior to the present invention, methods for obtaining a biologically active, recombinant CTLA4 protein involved expressing CTLA4 as a fusion protein. More particularly, the fusion protein is described in PCT Publication No. WO 93/00431 as containing the extracellular domain of CTLA4 fused to the heavy chain region of a human immunoglobulin molecule (referred to as "CTLA4-Ig" protein). According to this publication, successful expression of the extracellular domain of the CTLA4 receptor protein requires an expression system that permits the protein to form dimers. In contrast, the unfused or truncated versions of the CTLA4 protein appear not to be expressed in an active form. The publication further indicates that the Ig portion of the CTLA4-Ig fusion protein is believed to facilitate dimer formation and to aid in the purification of the fusion protein by conventional protein A affinity chromatography.

The present invention is based on the discovery of methods for producing a biologically active, soluble recombinant CTLA4 polypeptides (sCTLA4) that are not Ig fusion proteins. As used herein, the term "biologically active" refers to polypeptides that exhibit B7 binding activity.

The recombinantly-produced CTLA4 polypeptides of the invention have, as a basic unit, a monomer that consists essentially of the extracellular domain of the wild-type CTLA4 receptor protein. The monomers consist essentially of the amino acid sequence of SEQ ID NO:2. Monomers expressed in prokaryotic hosts cells, such as *E.Coli*, are encoded by an amino acid sequence similar to SEQ ID NO:2, but with a methionine at the N-terminal end. In reference to monomers, the term "consists essentially of" as used herein is intended to encompass a monomer encoded by an amino acid sequence corresponding to the extracellular domain of the wild-type CTLA4 protein or corresponding to the extracellular domain joined to additional amino acids other than an amino acid sequence encoding for a human Ig molecule. The calculated molecular weight of the CTLA4 monomeric form is about 12.5-13.5 kDa. The recombinantly

produced sCTLA4 monomer appears as two major bands in the range of about 14-16 kDa on SDS PAGE under non-reducing conditions.

The recombinant CTLA4 polypeptides of the present invention can also be in the form of dimers or other multimers, which contain more than one basic monomeric unit. Such multimers, particularly dimers, can be formed by joining two or more monomers through intermolecular disulfide bonds or by cross-linking agents such as, for example, polyethylene glycol (hereinafter referred to as "PEG"), other polyethers, EDTA and other linkers known to those skilled in the art. The dimeric form produced by two monomers joined by intermolecular disulfide bonds has a calculated molecular weight of about 25 kDa and appears as at least three major bands in the range of about 24-27 kDa on SDS PAGE under non-reducing conditions. The invention provides methods for separating the various dimer forms and purifying the most active dimer form.

The monomeric and dimeric forms are biologically active according to the assays described in the examples below. The active dimeric form, however, was found to be about 10- to 100-fold more active than the monomeric form of CTLA4 in these *in vitro* biological assays.

The present invention further provides methods of producing the recombinant sCTLA4 polypeptides. Such methods include the steps of:

(a) obtaining a DNA sequence capable of directing a host cell to express a polypeptide corresponding to the extracellular domain of a CTLA4 receptor protein, the polypeptide having B7 binding activity;

(b) inserting the DNA sequence into a vector having operational elements for expression of the DNA sequence;

(c) transferring the vector into a host cell capable of expressing the polypeptide;

(d) culturing the host cell under conditions for expression of the polypeptide;

(e) harvesting the polypeptide; and

(f) permitting the polypeptide to assume an active tertiary structure.

Optionally, the peptide can thereafter be permitted to assume a quaternary structure in which two or more monomers join to form a unit, such as a dimer or other multimeric forms. In addition, the present invention further optionally includes separating the dimeric forms to obtain the form with the most inhibitory activity, referred to herein as the active dimeric form.

The nucleic acid sequences useful in the present methods include SEQ ID NO:1 and its functional equivalents. As used herein, the term "functional equivalent(s)" means modified sequences having one or more additions, deletions, or substitutions to the above sequence that do not substantially affect the ability of the sequence to encode a polypeptide having B7 binding activity. Such modified sequences can be produced by means known in the art, including, for example, site directed mutagenesis. The sequences can be obtained from natural sources, such as the natural DNA sequence encoding the extracellular domain of a CTLA4 receptor protein. Alternatively, the sequence can be produced synthetically according to methods known in the art. Additionally, such DNA sequences can be derived from a combination of synthetic and natural sources. The natural sequences further include cDNA and genomic DNA segments. Methods of obtaining the synthetic and natural DNA sequences are described in PCT Publication No. WO 93/00431, published on January 7, 1993, which is incorporated herein by reference.

Vectors that can be used in these methods include those vectors into which a CTLA4 DNA sequence, as described above. As used herein, the term "consisting essentially of" in reference to vectors means that such vectors contain nucleotide sequences that encode for the extracellular domain of CTLA4 receptor protein, including any desired operational elements, but not nucleotide sequences encoding a human Ig molecule. A CTLA4 DNA sequence can be inserted and linked with any desired operational elements to effect its expression. The vectors can contain one or more of the following operational elements: (1) a promoter; (2) a Shine-Dalgarno sequence and initiator codon; (3) a terminator codon; (4) an operator; (5) a leader sequence to facilitate

transportation out of the host cell; (6) a gene for a regulator protein; and (7) any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vectors. EP Application No. 90 113 673.9, which is incorporated herein by reference, discloses several useful vectors and desirable operational elements.

The vectors can be transferred into suitable host cells by various methods known in the art, including transfection and transformation procedures. Various transfer methods are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. (1989), which is incorporated herein by reference. Such host cells can be either eucaryotic or procaryotic cells. Examples of such host cells include chinese hamster ovary (CHO) cells, yeast, *E. Coli* and baculovirus infected insect cells. The host cells described in EP Application No. 90 113 673.9, which is incorporated herein by reference, are also useful in the present methods.

The host cells of the present invention can be cultured under conditions appropriate for the expression of the recombinant CTLA4 polypeptide. These conditions are generally specific for the host cell and are readily determined by one of ordinary skill in the art in light of the published literature regarding the growth conditions for such cells. For example, Bergey's Manual of Determinative Bacteriology, 8th ed., Williams & Wilkins Co., Baltimore, Maryland, which is incorporated herein by reference, contains information relating to appropriate conditions for culturing bacteria. Similar information relating to culturing yeast and mammalian cells are described in R. Pollack, Mammalian Cell Culture, Cold Spring Harbor Laboratories (1975), incorporated herein by reference.

In one embodiment, cells can be grown to a high density in the presence of appropriate regulatory conditions that inhibit expression of the desired CTLA4 polypeptide. When optimal cell density is reached, the environmental conditions can be altered to those appropriate for expression of the polypeptide according to procedures known in the art or as described in the examples below. Therefore, prior to harvesting the expressed CTLA4

polypeptides, it is particularly useful to allow the host cells to grow near optimal density before inducing expression.

Expression of the recombinant CTLA4 polypeptides can be confirmed by using anti-CTLA4 antibodies according to assay procedures known in the art, such as Western blotting or ELISA for example. Once expression of the recombinant polypeptides has been confirmed, the polypeptides can then be harvested according to methods known to those skilled in the art.

The recombinant polypeptides can be purified after harvesting, and, if necessary, before or after allowing the recombinant polypeptide to assume an active structure. Preferably, the polypeptides are purified before assuming their active tertiary or quaternary structure. Methods for purifying the recombinant proteins are known in the art and include, for example, the methods described in EP Application No. 90 113 673.9, which is incorporated herein by reference.

For polypeptides that are expressed in a biologically inactive form or to increase their biological activity, the following general refolding procedures can be used. These procedures are particularly useful to produce biologically active polypeptides expressed by procaryotic host cells, such as *E. Coli*.

First, intramolecular or intermolecular disulfide bonds or other non-covalent interactions that have occurred during the expression of the CTLA4 polypeptides are disrupted by exposing the polypeptides to denaturing and reducing agents. Suitable denaturing agents are those compounds or chemicals that cause a change in the conformation of a protein by disrupting the intermolecular or intramolecular bonds that results in a loss of biological activity without substantially affecting its primary structure. Examples of such denaturing agents include guanidine hydrochloride and urea.

Preferably, guanidine hydrochloride is used as the denaturant. The concentration of guanidine is in the range of about 0.5M to about 6.0M, preferably at least about 6.0M.

If urea is used as the denaturing agent, any interfering cyanate that may form can be removed by passing the urea solution

over an anion exchange column, such as DOWEX 1-X8 (BioRad, Richmond, California). Cyanate can modify amino groups in the protein and, therefore, should be removed. (Stark, Methods in Enzymology 11:125 (1967))

Next, the disulfide bonds are then reduced with a reducing agent. Suitable reducing agents include, for example, beta-mercaptoethanol, dithiothreitol (DTT) and cysteine. Preferably, DTT is used as the reducing agent. The preferred DTT concentration is 6mM. In one embodiment as described in Examples 5 and 8A, the free thiols present in the reduced protein are oxidized by the addition of a large excess of an oxidizing agent, preferably a disulfide-containing oxidizing agent such as, for example, oxidized glutathione or cystine. Finally, the resulting solution is diluted prior to adding a second reducing agent to catalyze disulfide interchanges. Preferably, the second reducing agent contains a sulfhydryl (thiol) group such as, for example, DTT, 2-mercaptoethanol, dithioerythritol, cysteine, cystamine. The second reducing agent can also be a disulfide containing compound such as sodium borohydride or any of the Group VIA hydrides having added cystine, oxidized glutathione or any cysteine-containing peptides. The purpose of adding the second reducing agent is to produce an environment in which the CTLA4 recombinant polypeptides assume a variety of 3-dimensional configurations by the formation and breaking of various disulfide or other non-covalent bonds. Although not wishing to be bound by any particular theory, it is believed the proper 3-dimensional structure and disulfide bonding pattern of the wild-type CTLA4 receptor protein is energetically more stable than other possible conformations. Therefore, under conditions in which the recombinant polypeptides are allowed to assume a variety of 3-dimensional conformations, a significant proportion of the polypeptides will form biologically active conformations. In addition, this environment also facilitates the formation of dimers through intermolecular disulfide bonds.

In a second preferred method, the denatured and reduced protein is diluted and allowed to refold into monomers and dimers

without the addition of additional oxidizing or reducing agents as described in Example 8B.

The monomeric and dimeric forms can then be separated following the procedures described in Examples 5 and 8 below. Briefly, the mixture is first dialyzed and centrifuged. The resulting supernatant is thereafter passed over an ion, preferably an anion, exchange column, followed by passage over a sizing column, such as Superdex 75 column for example. Passage over the ion exchange column results in a dimer pool mixture of about 70% dimers, while the further passage over the sizing column results in a dimer pool mixture of at least 90% dimers, and preferably about 95% dimers. The same procedure yields a monomer pool mixture of at least about 85% monomers. Thereafter, the mixture can be passed over a phenyl sepharose column or, alternatively, over a reverse phase column for further separating the monomers from the dimers. Useful ion exchange columns include, without limitation, Mono Q, Q-Sepharose, Resource Q and Source 15Q columns. Other equivalent separation procedures known to those skilled in the art can also be used to separate the various recombinant CTLA4 forms.

The present invention also provides functional derivatives of the recombinant CTLA4 polypeptides. As used herein, the term "functional derivative" means any biologically active modified form of the recombinant CTLA4 polypeptides. Such modifications can be (1) substitutions or additions in the amino acid sequence, and/or (2) the addition of another functional group to be used as a cross-linking agent or to improve certain pharmacokinetic or immunologic properties. Such modifications, however, should not substantially decrease the biological activity of the parent recombinant polypeptide by no more than a 10-fold decrease, preferably less than a 5-fold decrease in activity. Therefore, as used herein, the term "functional derivative" can mean an active fragment, an analog or a derivative of a recombinant CTLA4 polypeptide described above that substantially retains the biological activity of the unmodified recombinant CTLA4 polypeptide. In the case of analogs, such modified polypeptides preferable have an amino acid homology of greater than about 40%

compared to SEQ. ID. NO. 2, more preferably in excess of 50%, and most preferably in excess of 90%. An amino acid homology of about 99% is particularly useful.

For example, one modification can be the substitution or addition of a cysteine to provide a "free cysteine" within the amino acid sequence to produce a "cysteine mutein." The terms "cysteine mutein" or "CTLA4 mutein," as used herein, refers to muteins having at least one cysteine that is not involved in an intramolecular or intermolecular disulfide bond. The free cysteine can appear at any amino acid residue that does not substantially interfere with its ability to bind B7. Preferably, a cysteine is substituted for at least one amino acid appearing at residue number 79, 80, 81, 109, 110, 111, or added after residue number 125 of SEQ.ID.NO. 2 from the N-terminal end.

The muteins and other derivatives can be prepared by methods well known to those skilled in the art. Such methods include, for example, mutagenic techniques in which nucleotides are substituted or added that encode for a cysteine. A general method is described, for example, in U.S. Patent No. 4,518,584, incorporated herein by reference. Alternatively, the muteins can be synthesized by methods also known to those skilled in the art.

In one embodiment, the cysteine mutein can be attached to polyethylene glycol (PEG) at a free cysteine to increase its molecular weight and improve its pharmacokinetic properties such as an increased serum half-life. Long chain polymer units of PEG can be bonded to the mutein via covalent attachment to the sulfhydryl group of a free cysteine residue on the mutein. Various PEG polymers with different molecular weights can be used, for example, 5.0 kDa (PEG_{5000}), 8.5 kDa (PEG_{8500}), 10 kDa ($PEG_{10,000}$), and 20 kDa ($PEG_{20,000}$). To obtain selectivity of reaction and homogenous reaction mixture, it is useful to use functionalized polymer units that will react specifically with the sulfhydryl groups. The functional or reactive group attached to the long chain PEG polymer is the activating group to which the mutein attaches at the free cysteine site. Suitable activating groups include, for example, maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane or 5-pyridyl. PEG

molecules can also be attached to CTLA4 at free amines using NHS (N-hydroxysuccinimide)-derivatized PEG molecules.

Other CTLA4 conjugates are also contemplated, for example, (1) by attaching a single PEG molecule to a CTLA4 monomer (mono-pegylated) or dimer, for example, at free amines as described in the examples below; (2) by attaching two PEG molecules to a CTLA4 dimer, or (3) by attaching two or more CTLA4 monomers or dimers through a cross-linking moiety, such as PEG, to produce a compound that can be depicted schematically as a "dumbbell." Alternatively, two or more CTLA4 dimers can be attached through a cross-linking moiety such as PEG to produce a "dimer dumbbell."

To create the dumbbell compounds, a PEG molecule containing two activating groups can be used such as, for example, PEG bis-maleimide (a PEG molecule containing a maleimide activating group on each end of the molecule) or bis-NHS-PEG (a PEG molecule containing an NHS group at each end of the molecule). Those skilled in the art can readily determine the appropriate pH, concentration of polypeptide, and ratio of polypeptide to PEG necessary to produce a useful yield of the mono-pegylated or dumbbell polypeptide.

The present invention further provides pharmaceutical compositions containing the recombinant CTLA4 polypeptides or its functional derivatives in a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" as used herein means a non-toxic, generally inert vehicle for the active ingredient, which does not adversely affect the ingredient or the patient to whom the composition is administered. Suitable vehicles or carriers can be found in standard pharmaceutical texts, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980), incorporated herein by reference. Such carriers include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline. In addition, the carrier can contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation.

The pharmaceutical compositions can be prepared by methods known in the art, including, by way of an example, the simple mixing of reagents. Those skilled in the art will know that the choice of the pharmaceutical carrier and the appropriate preparation of the composition depend on the intended use and mode of administration.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations can be stored either in a ready-to-use form or in a form that requires reconstitution prior to administration. Generally, storage of the formulations is at temperatures conventional for such pharmaceuticals, including room temperature or preferably 4°C or lower, such as -70°C. The formulations can be stored and administered between a pH range of about 5 to 8, preferably at about physiological pH.

The recombinant CTLA4 polypeptides and their functional derivatives can be used for a variety of purposes. In one embodiment, the recombinant polypeptides can be used as immunogens to produce polyclonal or monoclonal antibodies according to methods known in the art such as described, for example, in Harlow & Lane, Antibodies: A Laboratory Manual (1988), incorporated herein by reference. Because CTLA4 is immunosuppressive, preferably the recombinant CTLA4 polypeptides are first denatured and, if desired, reduced prior to their use as an immunogen to produce anti-CTLA4 antibodies. Such antibodies can, in turn, be used to detect CTLA4 receptor proteins on the cell surface of T cells or for *in vivo* uses such as imaging or to inhibit the binding of B7 to such receptor proteins according to procedures well known in the art.

The recombinant polypeptides of the present invention can also be used as research reagents to detect the presence of B7 or to purify B7 according to procedures known in the art. For diagnostic purposes, the recombinant polypeptides can be labelled with a marker prior to being exposed to a sample suspected of containing the ligand to be detected. The polypeptides can also be attached to a solid support for the purification of B7.

Additionally, the recombinant polypeptides and functional derivatives thereof can be used to prevent, suppress or treat disorders associated with inappropriate T cell activation and proliferation. Accordingly, the present invention provides methods for the therapy of disorders associated with such deleterious T cell activation and proliferation. Such disorders include, for example, transplantation rejection, various autoimmune diseases and other T-cell mediated disorders. PCT Publication No. WO 93/00431, incorporated herein by reference, describes various T-cell mediated disorders. The autoimmune diseases for which the administration of CTLA4 polypeptides and functional derivatives may be useful include rheumatoid arthritis, asthma, Lupus, multiple sclerosis, psoriasis, graft versus host disease, Type I diabetes and other autoimmune diseases described in E. Rubenstein & D. Federman, Scientific American Medicine, vol. 2, chapter IV (1993), incorporated herein by reference.

The therapeutic methods of the present invention are accomplished by administering to a patient an effective amount of a recombinant CTLA4 polypeptide of the present invention or a functional derivative thereof to inhibit deleterious T cell activation. The active ingredient is preferably formulated into a pharmaceutical composition as previously described.

As used herein, the term "patient" refers to any animal having T cells that are capable of being co-stimulated by B7, including humans. In addition, the recombinant CTLA4 polypeptides and their functional derivative are also referred to as the "active ingredient(s)."

An effective dosage depends on a variety of factors known to those skilled in the art, including the species, age, weight, and medical condition of the patient, as well as the type of disorder to be prevented, suppressed or treated, the severity of the condition, the route of administration and the active ingredient used. A skilled physician or veterinarian can readily determine and prescribe an effective amount of the active ingredient. Generally, treatment is initiated with small dosages substantially less than the optimum dose of the active

ingredient. Thereafter, the dosage is increased by small increments until the optimum or desired effect is attained without causing significant harm or deleterious side effects. Preferably, the daily dosage is in the range of about 10-2000 mg per human patient.

The compounds and pharmaceutical compositions of the present invention can be administered orally or parenterally by any means known in the art, including, for example, by intravenous, subcutaneous, intraarticular or intramuscular injection or infusion. To achieve and maintain the desired effective dose, repeated administration may be desirable. The frequency of dosing will depend on several factors such as, for example, the formulation used, the type of disorder, the individual characteristics of the patient, and the like. Those skilled in the art can readily determine the appropriate frequency based on such factors.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

Cloning of CTLA4

DNA sequences encoding the extracellular domain of the CTLA-4 protein were cloned from a human T cell leukemia cell line, Hut 78, using the Polymerase Chain Reaction (PCR) technique. The HuT 78 cell line (catalogue # TIB 161) was obtained from the American Type Culture Collection in Rockville, MD. The Hut 78 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 ug/ml streptomycin at $4x10^5$ cells/ml. The cells were activated by the addition of 5ng/ml phorbol 12-myristate 13-acetate (catalogue #P-8139, Sigma Chemical Company, St. Louis, MO), 1 µg/ml PHA-L (catalogue # L-4144, Sigma Chemical Company, St. Louis, MO), 5 ng/ml IL-2 (R&D Systems, Minneapolis, MN) and grown for an additional 49 hours. At harvest, $9x10^6$ cells were washed in phosphate buffered saline (PBS), pelleted, frozen

immediately in liquid nitrogen and stored at -70°C overnight. The next day the frozen cells were resuspended in 3ml of PBS and 1 ml ($3x10^6$ cells) was pelleted briefly in a microfuge. Messenger RNA was prepared from the cells using a "Micro FastTrack mRNA Isolation Kit" purchased from Invitrogen Corporation (San Diego,CA) according to the instructions provided by the manufacturer. The resulting mRNA pellet was resuspended in 10μl of water and 1ul was used to prepare first strand cDNA using a "cDNA Cycle Kit" (Invitrogen Corporation) and random primers supplied in the kit. The first strand cDNA synthesis procedure was performed according to the manufacturer's instructions.

The mature, extracellular portion (from alanine 1 to aspartate 125) of the cDNA for CTLA-4 (Dariavach et al., Eur. J. Immunol., vol. 18, pp. 1901-1905 (1988)) was amplified by PCR using one-fifth of the total cDNA volume (4 μl of 20 μl) of the first strand cDNA in a mixture containing 10mM Tris pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 200uM each of dATP, dCTP, dGTP, & TTP, and 20 pmoles of each oligonucleotide primer [5'CCCCATATGGCAATGCACGTGGCCCAGCCTGCT3' (SEQ ID NO:3) and 5'CCCAAGCTTGGTACCTTATCAGTCAGAATCTGGGCACGGTTCTGG3' (SEQ ID NO:4) (regions that overlap CTLA-4 DNA sequences are underlined) in a volume of 100μl. After denaturing the RNA/cDNA hybrids at 95°C for 1 minute the temperature was lowered to 60°C and 0.5μl (2.5 units) of "AmpliTaq DNA Polymerase" (Perkin-Elmer Corporation, Norwalk, CT) was added and the temperature raised to 72°C for 1 minute. The PCR was performed in an Ericomp "Twinblock" thermal cycler (San Diego, CA) with 29 additional cycles comprising 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C. The PCR amplification was completed with a 10 minute incubation at 72°C.

After verifying that a 0.4kb PCR fragment was produced (by running a small aliquot of the reaction mixture on a 1.5% agarose gel) the reaction mixture was extracted with phenol once, followed by precipitation with ethanol and subsequently digested with NdeI and HindIII restriction endonucleases. The digested DNA was put through a spin column to remove small DNA fragments and a small portion (equivalent to about one twentieth of the original PCR reaction) was ligated to NdeI-HindIII cut pT88IQ, a

Tac promoter expression plasmid, and inserted into E. coli host strain DH5-alpha (available from GIBCO BRL, Gaithersberg, MD).

The expression vector pT88IQ is a derivative of the expression vector pT3XI-2. The vector pT3XI-2 was constructed in the following manner. The starting plasmid for this construction was plasmid pKK223-3 purchased from Pharmacia. Plasmid pKK223-3 carries a partial gene for tetracycline resistance. This nonfunctional gene was replaced by a complete tetracycline resistance gene carried on plasmid pBR322. Plasmid pKK223-3 was digested completely with SphI and partially with BamH1. A 4.4 kilobase pair fragment was gel purified and combined with a synthetic adapter (SEQ ID NO:5):

```
5'  GATCTAGAATTGTCATGTTTGACAGCTTATCAT  3'
3'  ____ATCTTAACAGTACAAACTGTCGAATAGTAGC  5'
    Bg1II                              ClaI
```

and a 539 basepair fragment of DNA from a ClaI, SphI digest of the tetracycline resistance gene of pBR322 (PL Biochemicals, 27-4891-01). The resulting plasmid was designated pCJ1.

Next, a XhoI linker purchased from New England Biolabs (Beverly, Massachusetts) was inserted into plasmid pCJ1's PvuII site to form plasmid pCJX-1. This insertion disrupts the rop gene which controls plasmid copy number. Next, an EcoRI fragment containing the lacI gene was purified from plasmid pMC9 (Calos et al., 1983), then inserted into the XhoI site with XhoI to EcoRI adapters. The polylinker region in plasmid pKK223-3 was next replaced with a polylinker containing additional sites by cutting with EcoRI and PstI (SEQ ID NO:6):

```
5'  AATTCCCGGG TACCAGATCT GAGCTCACTA GTCTGCA  3'
3'  GGGCCC ATGGTCTAGA CTCGAGTGAT CAG  5'
```

The plasmid vector so obtained is designated pCJXI-1.

Finally, the tetracycline resistance gene was replaced with a similar gene which had the recognition sites for restriction enzymes HindIII, BamH1, and Sa1I destroyed by bisulfite mutagenesis. The following procedure was used to mutate the tetracycline resistance gene of pBR322. Plasmid pBR322 was cut with HindIII, then mutagenized with sodium bisulfite (Shortle and Botstein, 1983). The mutagenized DNA was ligated to form

circular DNA, then cut with HindIII to linearize any plasmid that escaped mutagenesis. This digestion mixture was used to transform *E. coli* JM109 (Yanisch-Perron et al., 1985). Tetracycline-resistant colonies were isolated and checked for loss of the HindIII site in the tetracycline resistance gene of the plasmid. A successfully mutated plasmid was designated pT1. A similar procedure was followed to mutagenize the BamH1 site in pT1, yielding plasmid pT2. Plasmid pT2 in turn was mutagenized to remove the SalI site, forming plasmid pT3. A ClaI-StyI fragment of pT3 carrying the mutated tetracycline resistance gene was isolated and used to replace the homologous fragment of pCJXI-1 to form pT3XI-2. The mutated tetracycline resistance gene still encodes for a functional protein. Downstream of the tac promoter region, a polylinker was introduced which contains, among other sites, BamH1 and KpnI restriction sites useful for cloning genes for expression in *E. coli* as described below.

As in pT3XI-2, the expression of the cloned gene containing the pT88IQ vector is driven by the tac promoter. Translation starts at the ATG of the unique NdeI recognition sequence CATATG (a downstream NdeI site was eliminated so that this start site NdeI sequence would be unique). There is a polylinker downstream of the NdeI site to facilitate insertion of the desired gene. In addition, the XhoI fragment containing the lacI region is replaced by a truncated fragment which eliminates the lacZ promoter and the operator region which is a binding site for the lac repressor. The lacI region in the replacement also carries the lacIq mutation -- a single base substitution which results in an increase in lac repressor production (Muller-Hill et al., Proc. Nat'l Acad. Sci. (U.S.A.) 59:1259-1264 (1968)).

The specific differences between pT3XI-2 and pT88IQ are as follows:

1. The cloning site region.

Between the EcoRI site upstream of the polylinker and the HindIII site at the downstream end of the polylinker, the following 135-mer sequence was substituted (SEQ ID NO:7):

5' >CACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA

CATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCCCGGGTACCGTCGA
CGAGCTCTTCGAACTAGTCCGCGGT > 3′

This sequence contains an NdeI site (underlined) at the start codon for expression and a polylinker containing recognition sites for BamHI, XmaI, KpnI, SalI, SacI, BstBI, SpeI and SacII.

2. The downstream NdeI site.

There is an NdeI site in pT3XI-2 about 2.4 Kb downstream of the cloning region. This site was eliminated so that the NdeI site at the start codon as described above was unique in pT88IQ. The site was changed from 5′ > CATATG > 3′ to 5′ > CATATATG > 3′, eliminating the NdeI recognition sequence.

3. The lacIq region.

The region in pT3XI-2 between the two XhoI sites containing the lacI region was replaced by the 1230 base sequence shown below:

lacIq sequence of pT88IQ (1230 BP) (SEQ ID NO:8)

CCATGGCTGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC
TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG
AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCCAGGGTG
GTTTTTCTTT TCACCAGTGA GACGGGCAAC AGCTGATTGC CCTTCACCGC
CTGGCCCTGA GAGAGTTGCA GCAAGCGGTC CACGCTGGTT TGCCCCAGCA
GGCGAAAATC CTGTTTGATG GTGGTTGACG GCGGGATATA ACATGAGCTG
TCTTCGGTAT CGTCGTATCC CACTACCGAG ATATCCGCAC CAACGCGCAG
CCCGGACTCG GTAATGGCGC GCATTGCGCC CAGCGCCATC TGATCGTTGG
CAACCAGCAT CGCAGTGGGA ACGATGCCCT CATTCAGCAT TTGCATGGTT
TGTTGAAAAC CGGACATGGC ACTCCAGTCG CCTTCCCGTT CCGCTATCGG
CTGAATTTGA TTGCGAGTGA GATATTTATG CCAGCCAGCC AGACGCAGAC
GCGCCGAGAC AGAACTTAAT GGGCCCGCTA ACAGCGCGAT TTGCTGGTGA
CCCAATGCGA CCAGATGCTC CACGCCCAGT CGCGTACCGT CTTCATGGGA
GAAAATAATA CTGTTGATGG GTGTCTGGTC AGAGACATCA AGAAATAACG
CCGGAACATT AGTGCAGGCA GCTTCCACAG CAATGGCATC CTGGTCATCC
AGCGGATAGT TAATGATCAG CCCACTGACG CGTTGCGCGA GAAGATTGTG
CACCGCCGCT TTACAGGCTT CGACGCCGCT TCGTTCTACC ATCGACACCA
CCACGCTGGC ACCCAGTTGA TCGGCGCGAG ATTTAATCGC CGCGACAATT
TGCGACGGCG CGTGCAGGGC CAGACTGGAG GTGGCAACGC CAATCAGCAA
CGACTGTTTG CCCGCCAGTT GTTGTGCCAC GCGGTTGGGA ATGTAATTCA

GCTCCGCCAT CGCCGCTTCC ACTTTTTCCC GCGTTTTCGC AGAAACGTGG
CTGGCCTGGT TCACCACGCG GGAAACGGTC TGATAAGAGA CACCGGCATA
CTCTGCGACA TCGTATAACG TTACTGGTTT CACATTCACC ACCCTGAATT
GACTCTCTTC CGGGCGCTAT CATGCCATAC CGCGAAAGGT TTTGCACCAT
TCGATGGTGT CGGAATTAAT TCAGCCATGG

This substituted region eliminates the lacZ promoter and the operator region which is a binding site for the lac repressor. It also contains the lacIq mutation which causes an increase in lac repressor synthesis (Muller-Hill et al., supra.).

Plasmid DNA was prepared from several of the resulting colonies and the inserted DNA was sequenced. Several clones of the expected sequence were found and expression studies showed that they produced a recombinant protein of the expected size (about 14 kDa) of sCTLA-4.

To increase the expression level, the sCTLA-4 region of clone 59-8-7 was cut out with NdeI and KpnI, eluted from an agarose gel to purify it away from the pT88IQ plasmid, and ligated to similarly cut T7 promoter expression vector pT5T as described in PCT Patent Publication No. WO 91/08285, incorporated herein by reference. The pT5T::sCTLA4 construct was inserted into *E. coli* host strain HMS174/DE3 (obtained from Dr. F. William Studier, Brookhaven National Laboratory, Upton, NY), and plasmid DNA prepared from 3 colonies. The plasmid DNAs were sequenced to verify that the sCTLA4 DNA sequences were correct and had been inserted correctly. One clone, 59-8-14, was selected for expression and refolding studies and is referred to henceforth as pT5T::sCTLA-4. All of the CTLA4 cDNAs obtained in the present study contained a threonine residue, and not an alanine residue, at amino acid position 111 of the protein sequence (SEQ ID NO:2). This result confirms the corrected nucleotide sequence of human CTLA4 reported by Linsley et al., J. Exp. Med. 174:561-569 (1991).

Preliminary expression of the sCTLA4 protein was performed by growing pT5T::sCTLA-4 in HMS174/DE3 in Luria Broth containing 12ug/ml tetracycline to an OD_{600} of 1.0 and inducing expression of sCTLA-4 by adding isopropyl beta-D thiogalactopyranoside (IPTG, catalogue #I-5502, Sigma Chemical Company, St. Louis, MO) to a

concentration of 1mM. Cells were harvested at two hours post-induction. Small aliquots of whole cells were boiled in SDS sample buffer containing 5% 2-mercaptoethanol for 2 minutes and run on a 14% polyacrylamide SDS gel. The most prominent band visible upon staining the gel with coomassie blue was approximately the 14 kDa sCTLA-4. This band was absent in a control culture lysate prepared from HMS174/DE3 that contained pT5T with a different gene (interleukin-6) in it.

EXAMPLE 2

Large Scale Production of sCTLA4

A 10 liter fermentation was done to provide sufficient quantities of recombinant sCTLA4 for biochemical analyses. *E.coli* strain HMS174/DE3 containing plasmid pT5T::sCTLA4 was grown at 37°C in 10 L of complex medium (40 g/L NZ-amine HD, 4 g/L KH_2PO_4, 1 g/L $MgSO_4$-$7H_2O$, 1 g/L Na_2SO_4, 0.3 g/L Na_3citrate-$2H_2O$, 50 g/L glycerol, 10 mg/L thiamine HCl, 2 ml/L trace minerals, 0.05 ml/L Mazu DF-204 and 15 mg/L tetracycline) until the optical density at A660 was 10. At this time the cells were induced by adding 0.24 grams of IPTG (a final concentration of 0.1 mM) to the culture. The cells were grown for an additional 6.5 hours and then harvested by centrifugation. The cell pellet was stored frozen at -20°C until use.

EXAMPLE 3

Preparation of Washed Inclusion Bodies (WIBS)

403 grams wet weight of the *E. coli* cell pellet prepared according to Example 2 were diluted with 2 liters of breaking buffer (25mM NaCl, 50mM Tris-HCl pH 7.5, 1mM dithiothreitol). The resulting slurry was passed through a Rannie mini-mill (APV Gaulin, Inc., Everett, MA) three times at a pressure of 10,000 PSI to break the cells. The broken cells were spun at 5,000 rpm in a Beckman J2-20 centrifuge using a JA-10 rotor for 15 minutes. The supernatant was decanted and discarded. The loose pellets were resuspended in breaking buffer and spun again for 60 minutes at 10,000 rpm and the supernatant decanted. A two-phase pellet

was observed: the lower pellet was white and the loose upper pellet was beige. The pellets were frozen at -20°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of samples from the two pellets showed that the majority of the sCTLA-4 was present in the lower white pellet and that the upper beige pellet was composed primarily of *E. coli* membrane proteins.

To remove the *E. coli* membrane proteins, the frozen pellets of Example 2 were thawed and resuspended in 1.5 liters of breaking buffer by homogenization with a Polytron PT 3000 mixer (Kinematica AG, Littau, Switzerland) at 8000 rpm. The mixture was then spun at 8000 rpm (11,000 x g) in a JA-10 rotor for 30 minutes. The pellets were frozen at -20°C. The next day the pellets were thawed and resuspended (by homogenization with the Polytron mixer at 5000 rpm) in 2.1 liters of breaking buffer and centrifuged at 8000 rpm as before. The bottles were decanted to remove most of the remaining beige membrane layer, which was discarded. The pellet that remains is referred to as washed inclusion bodies (WIBS). The wet weight of the washed inclusion bodies was 92.5 grams, which is about 23% of the weight of the original *E. coli* cell pellet. The WIBs were frozen at -20°C until used.

EXAMPLE 4

Refolding and in vitro activity of sCTLA4 from WIBS.

One gram of WIBs was denatured in 60 ml of freshly made 6M guanidine HCl, 0.1M Tris pH 8.0, 6mM dithiothreitol by homogenizing briefly using a Polytron mixer at 2000-3000 rpm and leaving at room temperature for 15 minutes. The insoluble debris was pelleted by spinning at 18,000 rpm in a JA-20 rotor for 15 minutes. 3.3 ml of 0.5M glutathione was added to the supernatant and left for 15 minutes at room temperature. The following solutions were added sequentially with stirring at room temperature:

1) 40 ml 6M guanidine in 50mM Tris-HCl, pH 9.7
2) 500 ml 50mM Tris-HCl, pH 9.7

3) 6 ml 0.5M cysteine

4) 6 ml 100mM phenylmethanesulfonyl fluoride (in 100% ethanol)

The preferred concentration of guanidine-HCl in the refold mixture was determined to be between 0.6M and 4M.

The bottle containing the sCTLA4 refold mixture was left at 4°C for 2 days to allow the sCTLA4 to refold into its proper conformation. At this time, 50 ml of the refold mixture was removed and tested for biological activity. Before testing, the refold mixture was centrifuged for 15 minutes in a JA-20 rotor at 8,000 rpm in a J2-21 centrifuge (Beckman Instruments, Palo Alto, CA) to remove any precipitate that had formed during the refolding procedure. Twenty-five ml of the supernatant was then dialyzed against 4 liters of 50 mM NaCl, 20 mM Tris-HCl, pH 8. Ten ml of human serum albumin (HSA) at a concentration of 300 ug/ml in 50 mM NaCl, 20 mM Tris-HCl pH 8 was dialyzed in the same flask. The HSA was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, catalogue number 823011). After dialysis the refold mixture and HSA solutions were centrifuged at 8,000 rpm for 15 minutes in a JA-20 rotor to remove precipitated material. The protein concentrations of the dialyzed sCTLA4 refold mixture and HSA were determined to be 750 ug/ml and 220 ug/ml, respectively.

The dialyzed, sCTLA4 refold mixture was tested for biological activity in an <u>in vitro</u> mixed lymphocyte reaction (see Methods in Immunology, pg.487-497, eds: J. S. Garvey, N. E. Cremer and D. H. Sussdorf, The Benjamin/Cummings Publishing Company, Reading, MA, 1977). In this assay lymphocytes from two different individuals are mixed together. Because of differences in their antigenicity, the cells recognize each other as foreign. This initiates an immune response that results in lymphocyte proliferation. The proliferative response of the cells is measured by pulsing the cells with 3H-thymidine according to the standard mixed lymphocyte reaction procedure described herein.

Lymphocytes were isolated from anticoagulated blood (treated with one-tenth volume of 3.8% sodium citrate made up in

an endotoxin-free 0.9% saline solution) obtained from human subjects A and B using Accuspin-System Histopaque 1077 media purchased from Sigma Diagnostics, St. Louis, MO. The cell isolation procedure followed was that described in the manufacturer's directions that accompany the kit. Lymphocytes from individual B were treated with mitomycin C (obtained from Sigma Chemical Company, St. Louis, MO) at a concentration of 25 ug/ml for 30 minutes at 37°C. The cells were washed four times with 10 ml of media to remove the mitomycin C. Lymphocytes from each individual were resuspended at 1 x 10^6/ml in Complete medium. (RPMI 1640 medium containing 25 mM HEPES buffer, 10% human AB serum, 2 mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin). In cases where the two lymphocyte populations were mixed, 100 μl of each cell suspension were added per well of a 96 well tissue culture plate (Corning Glass Works, Corning, NY). Control wells, which were used to measure proliferation of unstimulated lymphocytes, contained 200 μl of cells from a single individual. Aliquots of the dialyzed, unfractionated sCTLA4 refold mixture or HSA were suspended in complete medium at concentrations of 0.05-50 μg/ml. 50 μl aliquots of the suspensions were added to appropriate wells of the mixed cell populations. Samples were tested in triplicate. After incubation for 5 days at 37°C, each well received 2 μCi of ^{3}H-thymidine (Dupont, catalogue # NET-027Z) in 50 μl of complete medium. Approximately 18-20 hours later, the cells were harvested onto glass fiber filter strips using a PHD Cell Harvestor (both purchased from Cambridge Technology, Inc., Watertown, MA). The cells were washed 3 times with PBS and DNA precipitated using 7% trichloroacetic acid. Filters were washed with absolute methanol and air dried. ^{3}H-thymidine incorporated into DNA was measured by scintillation counting. Means of triplicate wells were calculated for each test sample.

The results of this experiment are shown in Table 1. The data show that the sCTLA4 refold mixture caused a dose-dependent inhibition of lymphocyte proliferation, as measured by a decrease in ^{3}H-Thymidine incorporation into DNA. The maximum inhibition observed was 85% at a protein concentration of 10 ug/ml. The

protein concentration of the refold mixture that gave 50% inhibition was about 100 ng/ml. HSA did not cause a similar inhibition of lymphocyte proliferation, indicating that the inhibition observed with the sCTLA4 refold mixture was specific.

TABLE 1

INHIBITION OF LYMPHOCYTE PROLIFERATION IN THE MIXED LYMPHOCYTE REACTION (HUMAN SUBJECTS A AND B) BY AN UNFRACTIONATED sCTLA-4 REFOLD MIX

DOSE (ng/ml)	REFOLD MIX		HSA	
	cpm*	% INHIBITION**	cpm*	% INHIBITION**
0	3535	0%	3036	0
10	2746	29%	3382	-17%
100	2153	51%	3694	-33%
1,000	1703	67%	3400	-18%
10,000	1237	85%	2953	4%

* Counts per minute. Means of triplicate wells.

** Percent inhibition was determined after first subtracting the cpms for an equivalent number of unmixed cells of subjects A and B from the cpm totals. These "unstimulated cell" cpms totaled 816 and 1021 for the sCTLA-4 refold mix and HSA, respectively. A negative percent inhibition indicates stimulation.

EXAMPLE 5

Purification and biological activities of sCTLA4 monomers and dimers

A. Purification

300 ml of a refold mixture prepared as described in Example 4 was dialyzed in SPECTRO/POR 3 tubing (Spectrum Medical Industries, Los Angeles, CA) against 5700 ml of 20mM Tris-HCl pH 8.0 at 4° overnight. After dialysis, an extensive precipitate was observed. SDS-PAGE analyses showed that the precipitate is composed largely of *E. coli* membrane proteins and improperly refolded sCTLA-4. The dialyzed refold mixture was centrifuged at 8000 rpm in a JA-10 rotor for 15 minutes. The supernatant was

passed through a 0.45 micron filter (Nalge Company, Rochester, NY) to remove residual precipitate and loaded onto a 20 ml Q-sepharose column (Pharmacia LKB, Picataway, NJ) and washed with 50mM guanidine, 20mM Tris-HCl pH 8.0. The bound protein was eluted with a 600 ml linear salt gradient from 0 to 60% 1M NaCl, 20mM Tris pH 8.0 at a flow rate of 5 ml/minute. Monomer forms of sCTLA-4 eluted at about 250mM NaCl (peak A) whereas dimer forms of sCTLA4 eluted at about 350mM NaCl (peak B). A broad protein peak eluting at about 450-600mM NaCl (peak C) also contained sCTLA4. This peak was composed primarily of sCTLA4 monomers and a few higher molecular weight forms of sCTLA4 which probably are disulfide crosslinked. This material may represent sCTLA4 forms that are aggregated. The monomeric and dimeric forms of sCTLA4 were confirmed by electrophoresing samples of the column fractions on 14% polyacrylamide SDS gels under non-reducing conditions. The dimeric form of sCTLA4 migrated as 2-3 bands in the 24-27 kDa molecular weight range. The monomeric form of sCTLA4 migrated as 2-3 bands in the 14-16 kDa molecular weight range. In the presence of a disulfide reducing agent (2-mercaptoethanol) both dimeric and monomeric sCTLA4 migrated as single bands with a relative molecular weight of about 15 kDa. These gel analyses indicated that the sCTLA4 dimers comprised two sCTLA4 proteins covalently attached to one another by a disulfide bond.

The column fractions containing sCTLA-4 peaks A,B and C were pooled separately and concentrated using a stirred cell concentrator and YM3 membranes (both obtained from Amicon, Inc., Beverly, MA). Pools A and B were concentrated to about 1.9 ml and pool C was concentrated to about 2.3 ml. The protein concentrations in the concentrated pools were about 900 ug/ml for pools A and B, and 1820 ug/ml for pool C. 1.5 ml of each concentrated pool was dialyzed in a multiwell dialysis manifold (BRL, Gaithersburg,MD) against 2 liters of 100mM NaCl, 20mM Tris-HCl pH 7.4 at 2.2 ml per minute at 4°C. Aliquots of the concentrated, dialyzed pools were examined on a non-reducing SDS gel. It was observed that pool A contained mostly sCTLA4 monomers and a small amount of sCTLA4 dimers; pool B contained mostly

sCTLA4 dimers and a small amount of sCTLA4 monomers; and pool C contained mostly sCTLA4 monomers but also contained some (approximately 10%) sCTLA4 dimers as well as smaller amounts of higher molecular weight forms of sCTLA-4, which probably are trimers, tetramers, etc. Five 200 μl aliquots of each pool were frozen and stored at -70°C until use in assays.

Aliquots (50 μl) of the monomer and dimer pools from the Q-Sepharose column (pools A and B, respectively) were loaded separately onto a Superdex-75 sizing column (3.2 x 300 mm; commercially available from Pharmacia LKB, Picataway,NJ) that had been equilibrated with 250 mM NaCl, 20 mM sodium acetate pH 5.5. The column was eluted with a flow rate of 50 μl/min. The monomer pool eluted as a major peak (70% of total protein) with a molecular weight of 15-17 kDa and as a minor peak (30% of total protein) with a molecular weight of 30-35 kDa. The dimer pool eluted as a major pool (70% of total protein) with a molecular weight of 30-35 kDa and a minor peak (30% of total protein) with a molecular weight of 15-17 kDa.

B. Hydrophobic Interaction Chromatography of CTLA4 Dimer

60 μl of the CTLA4 dimer pool (approx. 1.2 mg/ml) obtained from the Q-sepharose column was diluted up to 500 μl with 20 mM Tris-HCl, 2M NaCl, pH 7.4 and loaded to a 1 ml column packed with Phenyl Sepharose (Hi Sub)(Pharmacia/LKB, Pictaway NJ) previously equilibrated with 20 mM Tris-HCl, 2M NaCl, pH 7.4. Proteins were eluted with a linear gradient from 0-50% CH_3CN (and 2M-0M NaCl) in 30 min at a flow rate of 1.0 ml/min. sCTLA4 dimers eluted as a symmetrical peak at approximately 27% CH_3CN, 0.9M NaCl. sCTLA4 monomers eluted slightly later at about 30-35% CH_3CN, 0.8M NaCl.

C. Reverse Phase Purification of CTLA4

20 μl aliquots of the Q-sepharose monomer (Pool A) and dimer (Pool B) pools were diluted up to 200 μl (with 0.05% TFA in H_2O) and loaded separately to an RP-1 reverse phase column (SynChrom Inc.) previously equilibrated with 0.05% TFA in H_2O. Protein was eluted with a linear gradient from 0-100% CH_3CN (0.05% TFA) in 30 minutes at 100 μl/minute. The monomeric form of sCTLA4 eluted as

a single peak (approx. 95% pure) at approximately 85% CH_3CN. The dimeric form of sCTLA4 eluted as 5-6 heterogenous peaks at about 85-90% CH_3CN.

CTLA4 monomers and dimers obtained from the HPLC column were sequenced by the Edman degradation method known to those skilled in the art. The amino-terminal sequences obtained for monomers and dimers were identical: AlaMetHisValAla (SEQ ID NO:9). This sequence matches the amino-terminal sequence expected for sCTLA4, except that the N-terminal methionine residue is absent. The absence of the N-terminal methionine residue indicates that this residue is efficiently cleaved from the sCTLA4 protein by *E. coli* processing enzymes.

EXAMPLE 6

In vitro activity of recombinant sCTLA4 monomers and dimers

Aliquots of pools A,B and C from the Q-sepharose column described in Example 5 were tested for biological activity in a mixed lymphocyte reaction (procedure described in Example 4). The lymphocytes used for this experiment were isolated from human subjects C and D. Lymphocytes from subject D were treated with mitomycin C and washed as described in Example 4. The mixed lymphocyte cultures were set up as described in Example 4. Human serum albumin (HSA) was used as a control protein. The HSA for this experiment was prepared by mixing 4 mg of HSA in 60 ml of 20 mM Tris-HCl pH 8, 250 mM NaCl, 37.5 mM guanidine hydrochloride and concentrating the sample to 1.97 ml using a stirred cell concentrator and YM3 membranes (both obtained from Amicon,Inc., Beverly, MA). 1.2 ml of this mixture was dialyzed against 2 liters of 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 using a multiwell dialysis manifold (BRL, Gaithersburg, MD). The final protein concentration of the HSA pool was 1100 ug/ml. Recombinant sCTLA4 (aliquots of pools A (monomer), B (dimer) and C (late monomer) or HSA were suspended in complete medium at concentrations of 0.05-50 ug/ml. 50 μl aliquots added to appropriate wells of the mixed cell populations. Samples were tested in triplicate. After incubation for 5 days at 37°C, each well received 2 uCi of ^{3}H-

thymidine in 50 μl of complete medium. Approximately 20 hours later the cells were harvested as described in Example 4 and radioactivity incorporated into DNA determined by scintillation counting. The results of this experiment are given in Table 2.

TABLE 2

INHIBITION OF LYMPHOCYTE PROLIFERATION IN THE MIXED LYMPHOCYTE REACTION (HUMAN SUBJECTS C AND D) BY sCTLA-4 POOLS A, B AND C OBTAINED BY Q-SEPHAROSE COLUMN CHROMATOGRAPHY

DOSE	cpm*				% INHIBITION**			
(ng/ml)	POOL A	POOL B	POOL C	HSA	POOL A	POOL B***	POOL C	HSA
0	12,958	15,578	9,940	10,703	0	0	0	0
10	16,127	9,727	12,767	10,587	-35%	61% (48%)	-40%	2%
100	14,584	6,359	9,100	10,870	-18%	96% (75%)	12%	-2%
1,000	7,096	5,153	7,372	10,297	65%	109% (86%)	36%	5%
10,000	3,983	3,029	5,000	9,084	99%	131% (102%)	70%	22%

* Counts per minute. Means of triplicate wells. Pools A and B were tested on one 96 well plate and Pool C and HSA were tested on another plate.

** Percent inhibition was determined after first subtracting the cpms for an equivalent number of unmixed cells of subjects C and D from the cpm totals. These "unstimulated cell" cpms totaled 3907, 6002, 2847 and 3204 for sCTLA-4 pools A, B, C and HSA, respectively. A negative percent inhibition indicates stimulation.

*** Since the "unstimulated cell" cpm total for Pool B was greater than that for the other pools, percent inhibition for Pool B also was calculated using the mean (3319 cpm) of the "unstimulated cell" cpms for the other three test samples. The modified percent inhibition is shown in parenthesis in the Pool B column.

The data show that each of the Q-sepharose pools containing recombinant CTLA4 (pools A,B and C) caused a dose-dependent inhibition of lymphocyte proliferation in the mixed lymphocyte reaction, as evidenced by a decrease in radioactivity incorporated into DNA. No significant inhibition was seen with HSA, indicating that the inhibition was specific for sCTLA4. Pool B, which contained predominantly CTLA4 dimers, was a more

potent inhibitor of the proliferative response than was pool A (mostly CTLA4 monomers) or pool C (mostly aggregated CTLA4 monomers, dimers and higher molecular weight forms). The dose of pool B that inhibited lymphocyte proliferation by 50% was determined to be slightly less than 10 ng/ml. This number may be an overestimate of the potency of pool B because the counts per minute (cpms) of the unmixed lymphocyte cell populations used to calculate percent inhibition were higher in the pool B samples than in the other pools (see footnote to Table 2). To account for this, data for pool B also were calculated using the mean cpms of the unmixed cell populations of the other protein pools. These modified percent inhibitions are given in parentheses in Table 2. Using the modified figures, the dose of pool B that inhibited lymphocyte proliferation by 50% was determined to be about 10 ng/ml. Essentially complete inhibition of lymphocyte proliferation occurred at pool B protein concentrations of 1-10 ug/ml. The doses of pools A and C that inhibited lymphocyte proliferation by 50% were between 100 and 1,000 ng/ml for pool A and between 1000 and 10,000 ng/ml for pool C. Thus, the pool containing mostly CTLA4 dimers (pool B) had 10- to 100-fold greater specific inhibitory activity than did the pools containing mostly CTLA4 monomers or monomer aggregates (pools A and C).

Pools A, B and C were tested in a second mixed lymphocyte reaction experiment using lymphocytes obtained from human subjects E and F. Lymphocytes from individual F were treated with mitomycin C as described in Example 4. Other procedures of this experiment were as described in Example 4 and in the previous experiment. The results of this experiment are given in Table 3.

TABLE 3

INHIBITION OF LYMPHOCYTE PROLIFERATION IN THE MIXED LYMPHOCYTE REACTION (HUMAN SUBJECTS E AND F) BY sCTLA-4 POOLS A, B AND C OBTAINED BY Q-SEPHAROSE COLUMN CHROMATOGRAPHY

DOSE (ng/ml)	cpm*				% INHIBITION**			
	POOL A	POOL B	POOL C	HSA	POOL A	POOL B	POOL C	HSA
0	28,920	32,243	31,031	29,675	0	0	0	0
1	26,478	27,898	30,301	25,214	9%	15%	3%	16%
10	28,344	24,476	29,927	26,501	2%	26%	4%	12%
100	27,541	20,268	21,320	28,255	5%	40%	34%	5%
1,000	24,687	17,645	18,051	26,891	16%	49%	45%	10%
10,000	13,222	10,402	16,050	25,405	59%	73%	52%	16%

* Counts per minute. Means of triplicate wells. Pools A and B were tested on one 96 well plate and Pool C and HSA were tested on another plate.

** Percent inhibition was determined after first subtracting the cpms for an equivalent number of unmixed cells of subjects E and F from the cpm totals. These "unstimulated cell" cpms totaled 2372, 2280, 2400 and 2590 for sCTLA-4 Pools A, B, C and HSA, respectively.

As was seen in the previous experiment, pool B was more potent than pools A and C in inhibiting lymphocyte proliferation. The maximal inhibition seen with pool B in this experiment was 73%. The dose of pool B that inhibited lymphocyte proliferation by 50% was about 1 ug/ml. In contrast, the doses of pools A and C that inhibited lymphocyte proliferation by 50% were about 10 ug/ml. No significant inhibition of lymphocyte proliferation was seen with HSA.

None of the recombinant sCTLA4 pools completely inhibited lymphocyte proliferation in this experiment. There are several possible explanations for this. One explanation may be that the sCTLA4 proteins in pools A, B and C were inactivated during mixing of the samples . Another explanation may be that co-stimulatory molecules other than CTLA4's ligand, B7, were expressed on the surfaces of one subject's antigen-presenting cells. Other co-stimulatory molecules that are distinct from B7 are known to exist (Razi-Wolf et al., Proc. Nat'l Acad. Sci.

(U.S.A.) 89:4210-4214 (1992); Liu et al., Eur. J. Immunol., 22: 2855-2859 (1992). One subject had a white cell count that was about four times normal, suggesting that he had experienced an infection recently. Some of his white cells may have been activated as a consequence. That this may have been the case is suggested by the greater stimulation of lymphocyte proliferation seen in this experiment (11-14 times over the levels seen with unmixed cells) compared to the previous two experiments (3-4 times over the levels seen with unmixed cells). If one assumes that the 73% inhibition observed with pool B is the maximum inhibition that could have been achieved with sCTLA4 in this experiment, then the dose of pool B that gave 50% of this maximum inhibition was between 10 and 100 ng/ml, which is similar to 50% inhibitory doses determined in the previous two experiments.

EXAMPLE 7

Development of a Stable B7 Expressing Cell Line

As an alternative to the mixed lymphocyte reaction a new bioassay was developed that measures IL-2 production by a human T cell line in the presence of PHA lectin and a chinese hamster ovary (CHO) cell line that has been stably transformed to express the human B7 receptor protein. The IL-2 bioassay has several advantages over the mixed lymphocyte reaction. These advantages include the fact that the IL-2 bioassay takes only one and a half days to perform versus 6-7 days for the mixed lymphocyte reaction, the IL-2 bioassay is insensitive to bacterial endotoxin (which can contaminate sCTLA4 preparations prepared from bacteria), whereas the mixed lymphocyte reaction yields spurious data if endotoxin is present and the IL-2 assay uses cells lines rather than primary cells, which reduces the risk of infection and makes it easier to obtain large numbers of cells for assays. Development of the bioassay required cloning a human B7 cDNA, cloning it into a suitable vector for expression in eukaryotic cells and transforming and selecting CHO cells that express the B7 receptor protein.

A. Cloning of a human B7 cDNA

The B7 gene was cloned from the human Raji B cell line (ATCC No. CCL 86). mRNA was isolated from 3 x 10^6 Raji cells using a Micro-FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA copies of one tenth of the mRNA were made using a cDNA Cycle Kit (Invitrogen, San Diego). The B7 genes in one fifth of the Raji cDNA were amplified by PCR using oligonucleotide primers complementary to the B7 sequence 5' and 3' ends, Pfu Polymerase (Stratagene, San Diego), and a Gene Amp System 9600 Thermal Cycler (Perkin Elmer Cetus, CA).

The following oligonucleotide primers were used:

B7(5'p)32: 5' CCC AAG CTT TCA CTT TTG ACC CTA AGC ATC TG 3' (SEQ.ID.NO:10)

B7(3'p)36: 5' CCC TCT AGA TTA TAC AGG GCG TAC ACT TTC CCT TCT-3' (SEQ.ID.NO:11)

(overlaps with B7 sequence are underlined)

The PCR reaction mixture contained 20 mM Tris-HCl pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP, and TTP, 20 pmoles of each primer oligo, 4 μl of Raji cDNA, and 0.5 μl (1.25u) of Pfu polymerase (total volume = 50 μl). PCR conditions were 30 cycles of (1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C), followed by a 10 minute incubation at 72°C. After the PCR was completed, 45 μl of the reaction mixture was passed over a spin column (ChromaSpin-100, ClonTech, Palo Alto, CA), then 20 μl was digested with XbaI and HindIII and electrophoresed on a 0.8% agarose gel. The band of about 0.9kb was eluted and ligated to plasmid pRc/CMV (Invitrogen, San Diego, CA) that had been cut with the same restriction enzymes and gel purified in the same way. The ligation mixture was used to transform E. coli strain TOP10F'(Invitrogen, San Diego, CA). Colonies selected on Luria Broth agar plates containing 50 μg/ml of ampicillin were screened for plasmids containing inserts of the correct size and the inserted gene from one such construct was sequenced thoroughly on

both strands to verify that it had the expected sequence. This plasmid clone was named B7-5.

B. Creation of a Stable B7 Expressing Cell Line

Chinese Hamster Ovary cells (CHO-K1, ATCC No: CCL 61) were grown in DMEM and penicillin, streptomycin, glutamine, proline (20 μg/ml), and 10% fetal bovine serum. A titration experiment determined that 400 μg/ml of the antibiotic G418 (Genticin sulfate, GIBCO BRL, Gaithersburg, MD) was sufficient to kill the CHO cells. The pRc/CMV vector used to express the B7 gene contains an aminoglycoside phosphotransferase gene that confers resistance to G418. The B7-5 plasmid used to transfect the CHO cells was prepared using the Qiagen mini-prep procedure (Qiagen,Chatsworth,CA) in which 19.2 ml of overnight culture grown in Luria Broth with 50 μg/ml of ampicillin yielded 120 μl of plasmid DNA at about 1256 μg/ml. CHO cells were transfected by the calcium phosphate precipitate method using a kit from Invitrogen (San Diego, CA) according to the manufacturer's instructions. On day 4 after transfection, media was replaced with new media containing G418 at 400 μg/ml. On day 19 after transfection, G418-resistant cells were subjected to limiting dilution to select for individual resistant cells. Six individual colonies were isolated and subjected to a second round of limiting dilution. A single well of each was chosen for propagation. The cell lines were named F9, C11, G10, E12, C12 and H9.

C. FACS screening of transformed B7-CHO cell lines

The six G418-resistant cell lines were screened with an anti-human B7 monoclonal antibody to determine if they express B7 on their cell surfaces. Confluent T-75 flasks of each cell line were washed twice with 10 ml of calcium- and magnesium-free PBS and exposed to 10 ml of calcium- and magnesium-free PBS containing 10 mM EDTA for 10 minutes at room temperature. After pipeting up and down several times to loosen the cells, non-adherent cells were transferred to a 15 ml conical centrifuge tube and centrifuged at 1000 rpm in a GS-6R tabletop centrifuge

(Beckman instruments, Houston, TX). The cells were resuspended in 120 microliters ice cold FACS Media (RPMI 1640 media (Biowhittaker, Walkersville, MD, cat# 12-115B) containing 2% (v/v) fetal bovine serum and 0.1% sodium azide). The cell concentration was about 2X 10^7 per ml. Fifty microliters of each cell line were mixed on ice in a 1.5 ml microfuge tube with 50 microliters of FACS media containing a 1:1000 dilution of an anti-human B7 monoclonal antibody (Becton Dickinson, San Jose,CA, catalogue # 550024) or a control mouse IgG1 monoclonal antibody (Becton Dickinson, San Jose,CA, catalogue #550029). The cells were incubated with the antibody on ice for 55 minutes, the microfuge tubes filled with FACS media and then centrifuged 5 minutes at 300 x g in a microfuge. Supernatants were removed by aspiration and the cells resuspended in 100 microliters of ice cold FACS media containing a 1:25 dilution of FITC-labeled polyclonal goat anti-mouse antisera (Becton Dickinson, San Jose, CA, catalogue number 349031). The mixtures were incubated for 55 minutes on ice, the tubes filled with ice cold FACS media and centrifuged for 5 minutes at 300 x g in a microfuge. The supernatants were aspirated and the cells resuspended in 500 microliters of ice cold FACS media. Labeled cells were analyzed for positive fluorescence using a flow cytometer. Cell lines F9, C12 and H9 were positive for B7 expression using this assay.

D. IL-2 Production Bioassay

The F9, C12 and H9 cell lines were mixed with the CD28-positive human Jurkat T cell line (ATCC No. CRL 1863) in the presence of PHA-L lectin (Sigma Chemical Company, St. Louis, MO, Cat.# L-4144) to determine if they induced IL-2 production by the Jurkat cells. To each well of a 96 well tissue culture plate was added $1x10^5$ Jurkat cells and $5x10^4$ F9, C12, H9 or parent CHO cells. Control wells contained Jurkat cells only. PHA was added to each well to a final concentration of 10μg/ml. The final volume per well was 250 μl. Cells and chemicals were resuspended in IL-2 assay media (RPMI 1640 media (Biowhittaker, Walkersville, MD, cat# 12-115B) containing 25 mM HEPES buffer, penicillin, streptomycin, glutamine and 10% fetal bovine serum). The F9,

C12, H9 and parent CHO cells had been detached from their culture dishes by incubation in Dulbeccos calcium- and magnesium-free PBS containing 10 mM EDTA. The detached cells were washed several times in IL-2 assay media before counting and plating. Assays were performed in triplicate. After thorough mixing, the plates were incubated for approximately 20-24 hours at 37°C in a standard tissue culture incubator. The liquid in each well was then mixed by pipetting and 100 µl transferred to a new well of an IL-2 ELISA assay plate (R&D Systems Inc., Minneapolis, MN). The procedure used for the ELISA assay was that provided by the manufacturer except that IL-2 assay media was used as the blank. Optical density of the wells was read at 450nm - 570nm in a microplate plate reader (Molecular Devices, Menlo Park, CA). Optical density reflects the amount of IL-2 in the sample. Optical density means for the triplicate wells were calculated and converted to pg/ml IL-2 using an IL-2 standard curve. The results showed that the B7-CHO cell lines, F9, C12 and H9 cells induced production of 360 pg/ml, 300 pg/ml and 150 pg/ml IL-2, respectively. The parent, non-transfected CHO cells induced 20 pg/ml IL-2. Jurkat cells alone produced 20 pg/ml IL-2. These results indicated that the F9, C12 and H9 cell lines were capable of inducing IL-2 production by Jurkat T cells. The C12 cell line was chosen for further assay development. Control experiments showed that PHA was required to induce IL-2 production above background levels. Titration experiments showed that IL-2 production increased in a dose-dependent way with increasing numbers of C12 cells per well in the bioassay. The response was more logarithmic than linear. The standard IL-2 production assay used to measure bioactivities of sCTLA4 preparations uses 2.5×10^4 C12 cells, 1×10^5 Jurkat cells and 10 µl/ml PHA per well in the above bioassay. Since CTLA4 binds to and neutralizes B7 on the C12 cells, addition of CTLA4 to the test wells results in a decrease in IL-2 production, which is measured by a decrease in the optical densities of the test wells.

EXAMPLE 8

Identification and purification of properly refolded sCTLA4 dimers

As described in Example 5, most refolds of sCTLA4 contains multiple (typically at least three) dimer forms in the 24-27 kDa molecular weight range. The dimer species can be resolved by SDS-PAGE (14% non-reducing SDS gel) and by reverse-phase chromatography using a RP-4 column as described in Example 8B. The different dimer forms differ in their bioactivities in the IL-2 production assay. Only one of the dimer forms is capable of significantly inhibiting IL-2 production in this assay. The dimer form with the greatest specific activity probably is properly folded, whereas the less active dimer forms probably are misfolded. The different dimer forms could be separated from one another using a sizing column. The most active dimer species elutes latest from the column (smaller apparent molecular weight) As described in Example 8D and Table 6 below. "Active" and "less active" dimer species were separated from one another as described in the examples below. The active and less active dimer forms obtained using refold procedure 2 (described below) could be separated from one another using a Mono Q, Source 15Q ion-exchange columns or by using a phenyl-sepharose hydrophobic interaction column.

A. Refold Procedure 1

Thirty grams of WIBs was dissolved in 2000 ml of freshly made 6M guanidine HCl, 0.1M Tris pH 8.0, 6mM DTT using a polytron PT 3000 (Brinkman Instruments, Lucerne, Switzerland) and left for 15 minutes at room temperature. The solution was then centrifuged for 30 minutes at 10,000 rpm in a JA-10 rotor and the pellet discarded. To the supernatant was added 110ml of 0.5M glutathione (oxidized form). The solution was left for 15 to 30 minutes at room temperature and then slowly added to 18 liters of 0.44M guanidine HCl in 50mM Tris pH 9.7 with gentle stirring. 200 ml of 0.5M cysteine and 200 ml of 100mM phenylmethylsulfonyl fluoride (in ethanol) was then added. The refold mixture was left

at 4°C for 6 days without stirring. At that time the mixture was concentrated to about 1 liter using an S10Y3 spiral ultrafiltration cartridge (Amicon, Beverly, MA) and dialyzed in Spectra/Por 3 tubing (Spectrum Medical Industries, Houston, TX) against 19 volumes of 20mM Tris pH 8.0 for two to three days. The extensive precipitate was removed by spinning for 15 minutes at 10,000 rpm in a JA-10 rotor. Residual precipitate was removed by passing the supernatant through 0.45μm filters. The supernatant was loaded onto a 300ml Q-sepharose (Fast Flow; Pharmacia, Piscataway, N.J.) column at 10 ml per minute. Proteins were eluted from the column at 10ml/minute using a 0 to 60% gradient of 1M NaCl in 20mM Tris pH 8.0. Aliquots (25μl) of each fraction from the 30% to 40% region (approximately 300mM to 400mM NaCl) were electrophoresed on a non-reducing 14% SDS gel. When stained with coomassie blue R250 the active dimer species can be discerned as a tightly focused band whereas the inactive or less active dimer species ("less active dimers") are more diffuse. This distinction is readily apparent. The less active dimer species typically have slightly higher apparent molecular weights than the active dimer on a 14% non-reducing SDS gel. One less active dimer species often co-migrates with the active dimer species on SDS gels. This less active dimer species can be distinguished from the active dimer species using the sizing column described below. Q-sepharose column fractions containing the active dimer species were pooled and concentrated to about 40 ml using a YM-10 membrane in an Amicon stirred cell (Amicon,Beverly, MA). The concentrated active dimer pool was passed through a 7 liter, 85cm Sephacryl S-100 (Pharmacia) column in a buffer of 250mM NaCl, 20mM Tris pH 7.5 at 26 ml per minute. Aliquots (25μl) of each fraction across the main protein peaks (protein was detected by measuring absorbance at A_{280} nm of the fractions) were electrophoresed on a 14% non-reducing SDS gel as described above. The active dimer species elutes later than the inactive dimer species. This sizing column step removes most other contaminating proteins as well as most less active dimer species. Fractions that contain reasonably pure active dimer were pooled and concentrated to about 10ml using a YM-10 membrane

in a stirred cell (Amicon, Inc., Beverly MA). Protein concentrations were measured using a Lowry protein assay kit ("DC Protein Assay", Bio-Rad Laboratories, Richmond, CA) using IgG as the protein standard (Bio-Rad Laboratories, Richmond, CA).

B. Refold procedure 2

It was discovered that substantially more active CTLA4 dimer could be recovered from a refold mixture by modifying the refold procedure. Refold procedure 2 is an improved refold procedure that yields substantially more active CTLA4 dimer per gram of starting WIBS. Oxidized glutathione and cysteine, which are typically included in mixtures used to refold recombinant proteins from bacteria, are eliminated from the refold mixture, resulting in considerable cost savings. Oxidized glutathione and cysteine appear to interfere with the correct refolding of active CTLA4 dimers. It is believed that the oxidized glutathione and/or cysteine bind to cysteine residues in CTLA4 and interfere with protein refolding and/or correct disulfide bond formation. In refold procedure 2, no additional oxidizing or reducing agents are added to the refold mixture besides the DTT that was used originally to reduce the WIBS. The following is a typical refold starting with 30 g of WIBS.

Thirty grams of WIBs was dissolved in 600ml of freshly made 6M guanidine HCl, 50mM Tris pH 8.5 using a polytron PT 3000 (Brinkman Instruments) and then stirred slowly for 30 minutes at room temperature. 7.2ml of 0.5M DTT (final DTT concentration= 6mM) was added and the solution stirred slowly at room temperature for 1 hour. The solution was then centrifuged at 10,000 rpm for 30 minutes in a JA-10 rotor and the pellet discarded. The supernatant was slowly added to 11.4 liters of a guanidine/Tris solution, which contains 343.9 g of guanidine HCl, 10.9 g Tris-HCl, and 60.6 g of Tris base dissolved in 11.4 liters of water. The refold mixture was left at 4°C for 3 days. The solution was then passed through an SA-1 continuous flow centrifuge (Westfalia, Oelde, Germany) at 200 ml/min at 14 psi, followed by a chase with 2 liters of 0.6M guanidine HCl, 50mM Tris pH 9.5 at 4°C. The supernatant was concentrated to about 1

to 2 liters using a spiral ultrafiltration cartridge (S10Y3, Amicon Inc., Beverly, MA) and dialyzed in Spectra/Por 3 tubing (Spectrum Medical Industries, Houston, TX) against 19 liters of 20mM Tris pH 7.5 at 4°C for a day. The dialysis buffer was changed (same solution and volume) and dialysis continued for another day at 4°C. The extensive precipitate that formed was removed by centrifugation for 15 minutes at 10,000 rpm in a JA-10 rotor. Residual precipitate was removed by passing the supernatant through 0.45μm filters. The solution was loaded onto a 50ml Source 15Q (Pharmacia) column at 10 ml per minute. Proteins were eluted from the column at 10ml/minute with a 0 to 60% gradient of 1M NaCl in 20mM Tris pH 8.0. Aliquots (10μl) of each fraction from the major protein peak eluting at about 300mM NaCl were electrophoresed on a non-reducing 14% SDS gel. When stained with coomassie blue R250 the "active" dimer can be discerned as a tightly focused band, whereas the "less active" dimer bands are more diffuse. This distinction is readily apparent. In contrast to refold procedure 1, the less active dimers that form using refold procedure 2 typically have lower apparent molecular weights than the active dimer species on 14% non-reducing SDS gels. Fractions containing the active dimer were pooled and concentrated using a YM-10 membrane in a stirred cell (Amicon) to a volume of about 40 ml. The concentrated active dimer pool was passed through a 7 liter, 85cm Sephacryl S-100 (Pharmacia) column in 250mM NaCl, 20mM sodium acetate pH 5.5, at 26 ml per minute. Aliquots (10μl) of each fraction across the main protein peak (detected by absorbance at A_{280} nm) on a 14% non-reducing SDS gel, as described above. The active dimer elutes later than the less active dimers. This sizing column step removes most other contaminating proteins as well as most active dimers. Fractions containing predominantly active dimer were pooled and concentrated to about 10ml using a YM-10 membrane in a stirred cell (Amicon Inc., Beverly, MA). Protein concentration was determined using a Lowry protein assay kit ("DC Protein Assay", Bio-Rad Laboratories, Richmond, CA) using IgG (Bio-Rad Laboratories) as the protein standard.

The active dimer species obtained by the above procedure was further characterized by reverse phase HPLC. Aliquots (50µl-100µl) of the purified active CTLA4 dimer were diluted to 500µl with Buffer A (0.05% trifluoroacetic acid "TFA"), and injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom, Lafayette, IN), and eluted with 100% acetonitrile, 0.042% TFA (Buffer B) using a linear gradient (increase of 2.6% Buffer B/min) at a flow rate of 0.25 ml/min. The correctly refolded, active CTLA4 dimer eluted as a symmetrical peak at 27.8 min.

Table 4 compares yields of good dimer obtained from several refold experiments using procedure 1 and procedure 2. It is clear from the table that 4-5X as much active dimer was obtained from procedure 2 as was obtained using procedure 1.

TABLE 4

Experiment	Starting WIBS	sCTLA4 recovered
RF 16	30g	23mg
RF 17	30g	14mg
RF 19	30g	25mg
RF KC	36g	16mg
RF 22	30g	76mg
RF 23	30g	143mg
RF 24	30g	110mg
RF 25	30g	83mg

Experiments RF16, RF17, RF19 and RF-KC used refold procedure 1.
Experiments RF22, RF23, RF24 and RF25 used refold procedure 2.

C. Refold Procedure 3

In refold procedure 3, 0.4g WIBS was dissolved in 8 ml of 6M guanidine, 50 mM Tris-HCl, pH 8.5. Four ml of this solution was reduced by adding DTT to a final concentration of 6 mM. Two ml

of this solution was diluted 10-fold to 20 ml with 50 mM Tris-HCl, pH 9.5. The guanidine concentration was maintained at 1 M. The final DTT concentration was 0.6 mM. Protein was refolded for 3 days at 4°C. After dialysis against 20 mM Tris-HCl, pH 8, the protein was applied to a Mono-Q column (Pharmacia HR5/5 column) in a buffer of 20 mM Tris-HCl, pH 8. Protein was eluted with a linear gradient of 0-600 mM NaCl in 20 mM Tris-HCl pH 8 at a flow rate was 1 ml per min (an increase of 10 mM NaCl per min). The column profile showed a major protein peak eluting in fractions 32 and 33, followed by a smaller, broad protein peak eluting in fractions 34 to 37. SDS-PAGE analysis showed that the major protein species in fractions 32 and 33 co-migrated with the active dimer species obtained using refold procedure 1. Fractions 34 to 37 contained at least three dimer species, one of which (a minor component) co-migrated on the SDS gel with the active dimer species. The other two dimer species run slightly faster, i.e., lower apparent molecular weights, than the active dimer species. Fractions 35 and 36 were enriched for these faster migrating dimer species. To determine which dimer species had the greatest activity, fractions 32 and 33 were combined and fractions 35 and 36 were combined. The two pools were tested in the IL-2 production bioassay. Protein concentrations were determined by a Bradford assay using bovine serum albumin as the standard. Use of bovine serum albumin as the standard yields protein concentrations that are about half those obtained if IgG is used as the standard. The results (Table 5) show that the pool of fractions 32 and 33 had the greatest inhibitory activity, with an IC_{50} of about 100 ng/ml. This is comparable to the IC_{50} for the active CTLA4 dimer species purified using refold procedure 1. The pool of fractions 35 and 36 showed little titratable inhibitory activity and had an IC_{50} greater than 3 ug/ml.

TABLE 5

Protein (μg/ml)	Optical Density (450 - 570 nm)*	
	Pool 32/33	Pool 35/36
0	.319 ± .011	.276 ± .036
0.0015	.312 ± .013	.247 ± .020
0.0045	.307 ± .060	.242 ± .010
0.014	.222 ± .023	.257 ± .044
0.04	.208 ± .008	.241 ± .014
0.12	.173 ± .024	.232 ± .011
0.37	.118 ± .009	.210 ± .017
1.1	.117 ± .014	.193 ± .010
3.3	.056 ± .006	.177 ± .035
10	.053 ± .006	.114 ± .008

* Values are means ± one standard deviation for triplicate wells. Protein concentrations were determined using bovine serum albumin as the standard.

The major protein in fractions 32 and 33 is similar in molecular weight (by SDS-PAGE) and physical characteristics (elution time on ion-exchange columns) to the major protein in the main protein peak obtained using refold procedure 2. The less active dimer species in fractions 35 and 36 are similar if not identical to the improperly folded dimer species obtained using refold procedure 2.

D. Separation of active and less active dimer species on a sizing column.

In Experiment RF-KC, CTLA4 was refolded and dimers purified from the Q-Sepharose column essentially as outlined in refold procedure 1. At least three dimer species could be discerned by non-reducing SDS-PAGE. The correct or most active dimer species

constituted a minor amount of the total dimer species in this experiment. Fractions containing predominantly dimer species (Fractions 89-109) were pooled, concentrated to 222 ml, and applied to an S-100 sizing column as described in refold procedure 1. CTLA4 dimers eluted as a major protein peak, comprising fractions 33-41, followed by a smaller shoulder peak, comprising fractions 42-54. Non-reducing SDS-PAGE analysis showed that the shoulder peak was enriched for the active dimer species, although it did contain considerable amounts of the less active dimer species (at least 50% of the total protein in the shoulder peak). The major protein peak contained multiple dimer species that are predominantly the less active dimer species. The major protein peak contained little of the active dimer species. The main protein peak, fractions 33-41, were pooled (called Pool A) and tested for activity in the IL-2 production bioassay. Similarly, the shoulder peak, fractions 42-54, were pooled (called Pool B) and tested for activity in the bioassay. The results (Table 6) showed that only Pool B had significant inhibitory activity. The IC_{50} for Pool B was 120-370 ng/ml whereas the IC_{50} for Pool A was greater than 10 ug/ml. To further purify the active dimer species, Pool B, fractions 42-54, were pooled, concentrated to 40 ml, and reapplied to the S-100 column. Fractions were not collected until 1750 ml of buffer had flowed through the column. Collection of 25 ml fractions was then begun. The CTLA4 dimers eluted as two overlapping protein peaks: an early eluting peak, fractions 28 to 35, that contained multiple dimer species and a later eluting peak, fractions 36 to 44, that were enriched for a single dimer species. Pools from the two protein peaks were prepared, fractions 28-35 (Pool B-1) and fractions 36-41 (Pool B-2), and tested in the IL-2 production bioassay. The results (Table 6) showed that the late eluting peak (Pool B-2) was the most active, with an IC_{50} of between 120 and 370 ng/ml; the early eluting peak (Pool B-1) was much less active, with an IC_{50} of about 10 μg/ml.

TABLE 6

Protein (μg/ml)	Optical Density (450 - 570 nm)*			
	Pool A (Fractions 33-41)	Pool B (Fractions 42-54)	Pool B-2 (Fractions 36-41)	Pool B-1 (Fractions 28-35)
0	.556 ± .124	.556 ± .124	.298 ± .019	.341 ± .011
0.0015	.581 ± .021	.590 ± .039	.293 ± .011	.362 ± .065
0.0045	.528 ± .016	.515 ± .015	.272 ± .020	.383 ± .027
0.014	.463 ± .035	.579 ± .007	.243 ± .023	.305 ± .023
0.04	.573 ± .025	.472 ± .032	.196 ± .006	.379 ± .037
0.12	.520 ± .015	.424 ± .004	.134 ± .007	.348 ± .020
0.37	.568 ± .049	.298 ± .023	.154 ± .021	.271 ± .050
1.1	.514 ± .034	.247 ± .020	.088 ± .019	.256 ± .029
3.3	.421 ± .038	.201 ± .019	.039 ± .078	.177 ± .021
10	.375 ± .010	.099 ± .002	.067 ± .027	.169 ± .072

* Values are means ± one standard deviation for triplicate wells. The values for Jurkat cells alone were 0.089 ± .039, 0 ± .005 and 0 ± .006 for Pools A and B, Pool B-2 and Pool B-1 experiments, respectively.

E. Additional Purification of Refolded CTLA4

The CTLA4 dimer pool prepared substantially according to Refold Procedure 8B column, except purification was accomplished by using only an ion exchange column, was loaded directly onto a hydrophobic interaction column (HIC) (phenyl-sepharose, 5mm X 5cm, Pharmacia, Piscataway, NJ) previously equilibrated with 20 mM TRIS, pH 7.4, 250 mM NaCl. The bound protein was eluted with 30 column volumes using a linear gradient to 20mM TRIS, pH 7.4, 50% acetonitrile (CH_3CN), at a flow rate of 1 ml/min. CTLA4 eluted as two peaks: a major peak eluting at 15% CH_3CN, and a minor peak eluting at 25% CH_3CN. SDS-PAGE analysis demonstrated that the major peak corresponded to the correctly refolded, active CTLA4 dimer, while the minor peak corresponded to the mis-

folded, less active CTLA4 dimer species migrating with lower relative molecular weights.

EXAMPLE 9

Bioassay of different purified CTLA4 preparations

CTLA4 active dimers, purified using refold procedure 1, were assayed for activity using the IL-2 production assay described above. CTLA4 protein was diluted using IL-2 assay media to desired concentrations, mixed with 2.5×10^4 $B7^+$-CHO cells (C12 cells) and 1×10^5 Jurkat cells in the presence of 10 μg/ml PHA and incubated for approximately 24 hours at 37°C in a tissue culture incubator. Each protein dilution was assayed in triplicate using 96-well tissue culture plates (Corning, Corning, NY). Jurkat cells plus PHA were used as the control. The IL-2 concentrations of the wells was determined using an IL-2 ELISA kit as described above. The optical densities of the wells is proportional to the amount of IL02 in the wells, i.e., a higher optical density indicates higher IL-2 levels. The IC_{50}s (concentration to observe half-maximal inhibition of IL-2 production) of different CTLA4 dimer preparations ranged from about 100-300 ng/ml using this assay (Table 7).

TABLE 7

Protein (μg/ml)	Optical Density (450 - 570 nm)*			
	RF16	RF17	RF19	RF-KC
0	.324 ± .020	.407 ± .034	.292 ± .014	.298 ± .019
0.0015	.331 ± .005	.359 ± .058	.278 ± .022	.293 ± .011
0.0045	.278 ± .027	.226 ± .017	.274 ± .021	.272 ± .020
0.014	.290 ± .018	.220 ± .011	.243 ± .013	.243 ± .023
0.04	.207 ± .009	.231 ± .078	.250 ± .047	.196 ± .006
0.12	.151 ± .014	.128 ± .006	.160 ± .004	.134 ± .007
0.37	.129 ± .008	.088 ± .005	.143 ± .020	.154 ± .021
1.1	.075 ± .006	.064 ± .027	.095 ± .008	.088 ± .019
3.3	.070 ± .011	.023 ± .006	.081 ± .010	.039 ± .078
10	.059 ± .015	.033 ± .016	.059 ± .003	.067 ± .027

* Values are means ± one standard deviation for triplicate wells. The values for Jurkat cells alone were 0.035 ± .003, 0.206 ± .059, 0.027 ± .002 and 0 ± .005 for RF16, RF17, RF19 and RF-KC experiments, respectively. The Jurkat cell alone value was unusually high in the RF19 experiment for unknown reasons.

Active CTLA4 dimer prepared using refold procedure 2 had similar IC_{50}s in the IL-2 production bioassay.

EXAMPLE 10

Recombinant sCTLA4 Inhibits Cellular Damage In Animals

Tiegs et al. (Journal of Clinical Investigation vol. 90, 196, 1992) describe a T cell-dependent liver injury model in mice that is inducible by Concanavalin A (Con A). Con-A-induced liver damage is detectible within 8 hours and results from polyclonal activation of T cells by macrophages in the presence of Con A. Liver damage is measured by release of specific liver enzymes, including serum glutamate pyruvic transaminase (SGPT), into the

blood stream. The in vivo activity of sCTLA4 was assessed using this model. The CTLA4 protein was contained in a pool of refolds RF-16, RF-17, RF-19 and RF-KC prepared using refold procedure 1 (Table 7). Female Balb/C mice, 18-20g, were purchased from Charles River. The mice received an intravenous injection (15 mg/kg) of Con-A (Type V, catalogue # C-7275; Sigma Chemical Company, St. Louis, Missouri) at 0 hour and subcutaneous injections of saline or 0.3, 3 or 30 mg/kg CTLA4 at -2, 0, 2, 4, and 6 hours. At 8 hours, the mice were sacrificed and the serum levels of SGPT were measured using an Ektachem 700 analyzer (Kodak, Rochester, N.Y.). SGPT serum levels of individual mice are shown in Table 8.

TABLE 8

*Normal (no Con A)	sCTLA4 Dose (mg/kg/injection)†			
	0	0.3	3	30
118	932	3046	468	316
47	943	108	1981	1386
124	1673	783	525	286
295	420	972	211	260
85	2512	613	519	563
133	1917	1465	466	468
118	1163	4845	969	165
74	4914	1642	382	
77	2502			
57				
N = 10	N = 9	N = 8	N = 8	N = 7

* Normal control mice did not receive Con A
† Experimental mice received Con A plus the indicated doses of sCTLA4 numbers given are SGPT levels (mU/ml) for individual mice.

Mice receiving 3 or 30 mg/kg CTLA4 per injection showed a statistically significant decrease in SGPT levels, indicating less liver damage, compared to Con-A-only treated controls ($p<0.05$). An analysis of variance with Dunnett's multiple comparisons was performed to test for differences between the groups receiving saline and different doses of CTLA4.

EXAMPLE 11

PEGylation of Wild-type CTLA4

A. PEGylation with NHS-PEG Reagents

CTLA4 contains two lysine residues per monomer. Soluble CTLA4 can be efficiently PEGylated using an NHS-5K-PEG reagent, which preferentially reacts with free amines such as those present on lysine residues. The active CTLA4 dimer prepared according to Example 8E was concentrated to 1.5-4.0 mg/ml using a stirred pressure cell (Amicon, 50ml) containing a 3000 Da molecular weight cut-off (MWCO) membrane (YM3, Amicon). Refolded soluble CTLA4 was reacted with a 5,000 kDa NHS-ester polyethylene glycol (5K-NHS-PEG). The final reaction mixture contained 675 mg/ml, (28 mM) CTLA4, 9mM TRIS, 55mM sodium phosphate, pH 7.0, 396 mg/ml, (84 mM) 5K-NHS-PEG, 112mM NaCl. The molar ratio of PEG:CTLA4 was 3:1. The reaction was carried out at room temperature for 5-6 hours. The reaction mixture was stored at 4°C. SDS-PAGE analysis (non-reduced conditions) demonstrated that the PEGylated products consisted of a major and a minor product migrating with relative moleuclar weights of 46,000 Da, and 65,000 Da, respectively. The percent overall conversion of the CTLA4 starting material was approximately 50-60%.

B. Isolation of Lysine-PEGylated Products

The reaction mixture containing the PEGylated products was diluted with an equal volue of 20 mM TRIS, pH 8.0 (Buffer C), and loaded onto an anion exchange column (Resource Q, 5mm X 50mm, volume = 1.0 ml, Pharmacia, Piscataway, NJ) previously equilibrated with Buffer C. The bound protein (and unreacted PEG) were eluted with a linear gradient (20 column volumes) to 0.5 M NaCl at a flow rate of 1.0 ml/min. 0.5ml fractions were collected. The minor PEGylated product (migrating at 65000 Da on SDS-PAGE) eluted in fractions 9 and 10 at about 0.3M NaCl; the major PEGylated product (migrating at 46000 Da on SDS-PAGE) eluted in fractions 11 and 12 at about 0.35 M NaCl. Unreacted PEG and unreacted CTLA4 dimer eluted in fractions 14 and 15 at about 0.4M NaCl.

C. Analysis of PEGylated Products

Aliquots of both the major and minor PEGylated products, and unreacted CTLA4 dimer were analyzed by SDS-PAGE under both non-reducing and reducing conditions. Reduction of the unreacted CTLA4 dimers yielded monomers migrating with a relative molecular weight of 12000 Da. Reduction of the major PEGylated product (migrating with a relative molecular weight of about 46,000 Da by non-reducing SDS-PAGE) yielded both unPEGylated monomer and PEGylated product migrating at molecular weights of 12000 Da and 18000 Da, respectively, in a molar ratio of 1:1. This demonstrates that the major PEGylated product (46,000 Da) is a mono-PEGylated dimer, i.e. only one of the monomer subunits is PEGylated and it contains a single PEG molecule. The species migrating at 18000 Da after reduction is a mono-PEGylated monomer. Reduction of the minor PEGylated product (migrating with a relative molecular weight of 65,000 Da by non-reducing SDS-PAGE) yielded 1 major product: a PEGylated product migrating at 18000 Da. This 18,000 Da PEGylated product is mono-PEGylated monomer indicating that the 65000 Da PEGylated product is doubly PEGylated CTLA4 dimer. Since reduction of this doubly PEGylated species does not yield significant amounts of unPEGylated monomer, each CTLA4 monomer contains a single PEGylated lysine residue. Small amounts of both a PEGylated product migrating at 46000 Da and unPEGylated monomer were also detected after reduction of the 65,000 Da minor PEGylated product. It is likely that these are the products from small amounts of high MW (> 67000 Da) PEGylated species contaminating the 65,000 Da product.

D. Bioactivities of PEGylated Products

Fractions 9 and 10 (doubly PEGylated dimers), fractions 11 and 12 (mono-pegylated dimers) and fractions 14 and 15 (unreacted dimers) fro Example 11B were pooled separately, concentrated and assayed for activity in the IL-2 production bioassay described in Example 7. Results, shown in Table 9, indicate that mono-pegylated CTLA4 had an IC_{50} approximately 3-4X greater than that of unpegylated CTLA4 (400 ng/ml versus 100 ng/ml). The IC_{50} for

di-pegylated CTLA4 was about 6X greater than that of unpegylated CTLA4.

TABLE 9

Protein (μg/ml)	Optical Density (450 - 570 nm)*		
	Fractions 9/10	Fractions 11/12	Fractions 14/15
0	.522 ± .026	.575 ± .024	.597 ± .022
0.0015	.502 ± .004	.553 ± .032	.556 ± .025
0.0045	.486 ± .010	.531 ± .034	.582 ± .047
0.014	.443 ± .028	.552 ± .029	.499 ± .016
0.04	.423 ± .012	.452 ± .030	.391 ± .013
0.12	.415 ± .045	.383 ± .017	.307 ± .005
0.37	.395 ± .035	.325 ± .024	.225 ± .022
1.1	.292 ± .018	.228 ± .017	.114 ± .008
3.3	.219 ± .009	.140 ± .006	.075 ± .010
10	.152 ± .019	.094 ± .009	.057 ± .003

* Values are means ± one standard deviation for triplicate wells. The values for Jurkat cells alone were 0.026 ± .003, 0.049 ± .004 and 0.040 ± .017 for Fractions 9/10, Fractions 11/12 and Fractions 14/15 experiments, respectively. Protein concentrations were determined using bovine serum albumin as the standard.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

What is claimed is:

1. A CTLA4 polypeptide comprising (a) at least one recombinantly-produced monomer consisting essentially of the amino acid sequence of SEQ ID NO:2 or the amino acid sequence of SEQ ID NO:2 having a methionine at the N-terminal end, or (b) a functional derivative of said monomer, said CTLA4 polypeptide capable of binding to B7.

2. The CTLA4 polypeptide of claim 1, wherein said monomer consists essentially of the amino acid sequence of SEQ ID NO:2.

3. The CTLA4 polypeptide of claim 1, wherein said monomer consists essentially of the amino acid sequence of SEQ ID NO:2 having an N-terminal methionine.

4. The CTLA4 polypeptide of claim 1, wherein said functional derivative comprises a CTLA4 mutein having at least one free cysteine.

5. The CTLA4 polypeptide of claim 1, wherein a polyethylene glycol (PEG) molecule is attached to said CTLA4 polypeptide.

6. The CTLA4 polypeptide of claim 1, wherein said polypeptide is a monomer.

7. The CTLA4 polypeptide of claim 1, wherein said polypeptide is a multimer.

8. The CTLA4 polypeptide of claim 1, wherein said polypeptide is a dimer.

9. The CTLA4 polypeptide of claim 8, wherein said dimer comprises two CTLA4 polypeptide monomers cross-linked by a PEG molecule.

10. The CTLA4 polypeptide of claim 8, wherein said dimer comprises two CTLA4 polypeptide monomers joined by a disulfide bond.

11. The CTLA4 polypeptide of claim 1, wherein said polypeptide is recombinantly produced in a prokaryotic host cell.

12. A vector consisting essentially of a nucleotide sequence encoding the CTLA4 polypeptide of claim 1.

13. The vector of claim 12, wherein said nucleotide sequence contains operational elements to express the CTLA4 polypeptide.

14. The vector of claim 12, wherein said nucleotide sequence is SEQ. ID. NO 1.

15. A host cell comprising the vector of claim 12.

16. The host cell of claim 15, wherein said host cell is *E.Coli*.

17. A pharmaceutical composition comprising the CTLA4 polypeptide of claim 1 in a pharmaceutically acceptable carrier.

18. A method for producing the recombinant CTLA-4 polypeptide of claim 1, comprising:

(a) obtaining a DNA sequence capable of directing a host cell to express the recombinant CTLA4 polypeptide;

(b) inserting the DNA sequence into a vector having operational elements for expression of the DNA sequence;

(c) transferring the vector into a host cell capable of expressing the polypeptide;

(d) culturing the host cell under conditions for expression of the polypeptide;

(e) harvesting the polypeptide; and

(f) permitting the polypeptide to assume an active tertiary structure.

19. The method of claim 18, wherein guanidine is used as a denaturing agent in step (f).

20. The method of claim 19, wherein guanidine is used at a concentration of 0.5M to 4.0M.

21. The method of claim 18, further comprising after step (f) the step of permitting the polypeptide to assume an active quaternary structure to form a multimer.

22. The method of claim 21, wherein said multimer is a dimer.

23. A method for substantially separating monomeric and dimeric forms of the recombinantly-produced CTLA4 polypeptide according to claim 18, comprising:

(a) passing a mixture of said CTLA4 polypeptide forms over an ion exchange column; and

(b) passing the resulting mixture of step (a) over a sizing column to substantially separate the CTLA4 monomeric and dimeric forms.

24. A method for substantially separating active dimers from less active dimers of the recombinantly-produced CTLA4 polypeptide according to claim 18, comprising:

(a) passing a mixture of said CTLA4 polypeptide active and less active dimers over an ion exchange column; and

(b) passing the resulting mixture of step (a) over a sizing column or a hydrophobic interaction column to substantially separate the active dimers from less active dimers.

25. The method of claim 24, wherein said resulting mixture of step (a) is passed over said sizing column and said hydrophobic interaction column.

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/US 94/07685

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705 C12N15/62 C12N1/21 A61K38/17 //(C12N1/21, C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	EP,A,0 613 944 (BRISTOL-MYERS SQIBB COMPANY) 7 September 1994 see page 6, line 19 - page 6, line 33; example 2	1-18
X	WO,A,93 00431 (BRISTOL-MYERS SQUIBB CO.; US) 7 January 1993 cited in the application see page 11, line 14 - page 20, line 1; examples 2,3	1-18

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 1994	29 -11- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0613944	07-09-94	AU-B-	5390194	28-07-94
		CA-A-	2113744	23-07-94
		FI-A-	940270	23-07-94
WO-A-9300431	07-01-93	AU-A-	2240092	25-01-93
		CA-A-	2110518	07-01-93
		EP-A-	0606217	20-07-94
		PT-A-	100637	31-05-94